Neuropeptide signalling systems involved in the timing of puberty onset and regulation of the gonadotrophin-releasing hormone pulse generator in the rat

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Neuropeptide signalling systems involved in the timing of puberty onset and regulation of the gonadotrophin-releasing hormone pulse generator in the rat

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

(Neuroscience)

September 2015

ABSTRACT

The pulsatile release of luteinising hormone (LH) induced by intermittent release of the gonadotrophin-releasing hormone (GnRH) is essential for initiating puberty and maintaining normal reproductive functions. Many neuropeptides including neurokinin B (NKB), substance P, kisspeptin (Kiss1) and gonadotrophin-inhibiting hormone (GnIH) have been implicated in regulating the secretion of LH. However, few studies have demonstrated the effects of those neuropeptides on pulsatile LH release and the dynamic changes of LH secretion in different gonadal hormone conditions. Studies outlined in this thesis have shown that antagonism of NKB receptor inhibits the pulsatile release of LH and delays the timing of puberty onset in female rats. Furthermore, NKB receptor agonism suppresses pulsatile LH release in ovariectomised (OVX) adult rats, but stimulates LH secretion in OVX rats replaced with 17β-oestradiol (E_2). Likewise, intra-arcuate nucleus (ARC) administration of substance P, a neuropeptide belonging to the same family of NKB, suppresses pulsatile LH release in OVX rats, but stimulates LH secretion in OVX rats replaced with E_2.

Kiss1, a potent stimulator of the hypothalamo-pituitary-gonadal (HPG) axis, has been studied extensively within the hypothalamus, but little is known about its extra-hypothalamic effects. Current studies have shown that administration of Kiss1 into the medial amygdala (MeA), a key limbic brain structure involved in reproduction, stimulates LH secretion in OVX rats. Furthermore, intra-MeA administration of Kiss1 receptor antagonist inhibits the pulsatile LH release in

OVX rats and spontaneous LH surges in intact female rats. Unlike Kiss1, GnIH is a major inhibitor of the reproductive axis. Central administration of RFamide-related peptides-3 (RFRP-3), the mammalian orthologue of GnIH, suppresses the pulsatile LH release in OVX rats. This effect is partially mediated through the endogenous opioid peptides. RFRP-3 is also implicated in stress-induced suppression of pulsatile LH secretion. Furthermore, antagonism of RFRP-3 receptor in the ARC and the medial preoptic area (mPOA) stimulates LH secretion in OVX rats with E_2 replacement, indicating that RFRP-3 may also regulate the E_2-induced negative feedback control of LH release. Taken together, the present study has demonstrated that various neuropeptides signaling systems including NKB, substance P, Kiss1 and RFRP-3 regulate pulsatile LH secretion in female rats.

ACKNOWLEDGEMENTS

I am grateful to a number of individuals at King's College London for helping me complete this thesis. I would like to express my deepest gratitude to Prof. Kevin O'Byrne, my primary PhD supervisor, who encouraged me to pursue a higher degree in the UK and offered to mentor my research at King's. He has been extremely patient, understanding and supportive, especially in offering constructive advice on writing this thesis and papers. He has encouraged me to submit abstracts for presentation at both national and international conferences, and to submit papers for publication.

I would like to thank other members in my lab, especially Dr Xiaofeng Li for her invaluable help and support with a number of different techniques used in this project, particularly with regard to the animal surgeries and the automated blood sampling system. I would also like to thank her for her help and advice in many other aspects throughout my PhD. Also, I would like to thank Miss Minghan Hu, a same-year PhD student, for her collaboration throughout my PhD in various aspects of the work, including the animal surgeries and radioimmunoassay. Thanks also to all other members of the group including Dr Rebecca Gresham, Dr Pasha Grachev, Mr Isha Khan, Ms Chloe Manley, Ms Claire Cooke and Dr Yuanshao Lin for their help with the experiments.

I would also like to thank my secondary supervisor, Prof. Lucilla Poston, for providing me with invaluable mentorship, particularly with respect to reports

and publications. Further, I wish to thank Prof. Peter Hylands for interviewing me in Beijing and offering me the King's Overseas Studentship which funds my PhD. Also, I wish to thank the British Society for Neuroendocrinology and the Physiology Society for funding me to attend related conferences and summer schools. Finally, I would like to thank my parents for supporting me financially.

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1. Hu MH, Li XF, McCausland B, **Li SY**, Gresham R, Kinsey-Jones JS, Gardiner JV, Sam AH, Bloom SR, Poston L, Lightman SL, Murphy KG, O'Byrne KT (2015). Relative importance of the arcuate and anteroventral periventricular kisspeptin neurons in control of puberty and reproductive function in female rats. Endocrinology; 156(7): 2619-31.

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LIST OF ABBREVIATIONS

ARC	Arcuate nucleus
AVPV	Anteroventral periventricular area
BNST	Bed nucleus of the stria terminalis
CRF	Corticotrophin-releasing factor
DMH	Dorsomedial hypothalamic area
Dyn	Dynophin
E_2	17β-oestradiol
FSH	Follicle-stimulating hormone
GABA	Gamma-aminobutyric acid
GnIH	Gonadotrophin-inhibitory hormone
GnRH	Gonadotrophin-releasing hormone
GPR147	G protein-coupled receptor 147
HPA	Hypothalamo-pituitary-adrenal
HPG	Hypothalamo-pituitary-gonadal
icv	Intracerebroventricular
iv	Intravenous
Kiss1	Kisspeptin
Kiss1r	Kisspeptin receptor
Kp-10	Kisspeptin-10
LH	Luteinising hormone
LPS	Lipopolysaccharides
ME	Median eminence
MeA	Medial amygdala
mPOA	Medial preoptic area
MUA	Multiunit electrical activity
NKB	Neurokinin B
NK3R	Neurokinin 3 receptor
NMDA	N-methyl-D-aspartate
OVX	Ovariectomised
pnd	Postnatal day
PVN	Paraventricular nucleus
RFRP-3	RFamide related peptide-3

CHAPTER ONE: GENERAL INTRODUCTION

1.1 The hypothalamo-pituitary-gonadal axis

Reproduction is essential for the evolution and survival of all life forms on earth. This process is biologically complex, particularly in higher organisms such as mammals. The reproductive system of mammals is tightly regulated by the hypothalamo-pituitary-gonadal (HPG) axis. Generally, this axis consists of a feedback loop that incorporates hypothalamic neurones, endocrine cells of the anterior pituitary and the gonads, which are in communication via hormonal signals.

The major hypothalamic effector of the HPG axis is the neuropeptide gonadotrophin-releasing hormone (GnRH), which is secreted from a group of hypothalamic neurones. In rats, the majority of GnRH neuronal cell bodies are located in the medial preoptic area (mPOA) (Witkin *et al.* 1982). The GnRH neurones project to the median eminence (ME) and release GnRH in a pulsatile fashion into the pituitary portal blood system (Belchetz *et al.* 1978, Witkin *et al.* 1982). In the anterior pituitary gland, GnRH binds with GnRH receptors on specialised gonadotroph cells, which leads to the secretion of the gonadotrophins luteinising hormone (LH) and follicle-stimulating hormone (FSH) into the systemic circulation (Amoss *et al.* 1971, Schally *et al.* 1971a). GnRH pulse frequency determines LH pulse frequency but is not tightly coupled to that of FSH (Clarke and Pompolo 2005). Furthermore, basal levels of LH and the

amplitude of LH pulses are affected by the frequency of GnRH/LH pulses (Clarke and Cummins 1985). High frequency of GnRH or LH pulses lead to lower amplitude of LH pulses, whereas low frequency lead to higher amplitude (Clarke and Cummins 1985, Wu *et al.* 1987).

In females, gonadotrophins stimulate the synthesis of progesterone (P_4) and oestrogens, the most notable of which is 17β-oestradiol (E_2). The female HPG axis is subject to feedback stimulation or inhibition by these gonadal steroids produced mainly in the ovary. The female ovary is characterised by spontaneous cycles of ovarian follicle development, ovum selection and ovulation. After the initiation of puberty in the rat, a number of follicles commence growth every day to form a continuous source of developing follicles. The immature primordial follicles contain immature oocytes surrounded by a single layer of granulosa cell. At the beginning of each cycle this single cell layer proliferates to form the outer thecal cell layer and inner granulosa cell layer surrounding the developing oocytes. LH stimulates thecal cells via LH receptor to synthesize androgens, which are aromatized by granulosa cells to produce E_2 (Makris and Ryan 1977, Magoffin and Erickson 1982). This aromatization is stimulated by FSH. The E_2 can bind to receptors in the granulosa cells, which are then stimulated to proliferate and produce more E_2 (Chakravorty *et al.* 1993). This positive feedback of E_2 synthesis leads to high circulating levels of E_2, whch are stimulatory to the activity of the GnRH neural network. The resultant increase in GnRH secretion induces an LH surge (Sarkar *et al.* 1976), which triggers

domiant follicles to release ova, while others undergo atresia. The collapsed follicles are transformed into corpus lutea which secret P_4 and E_2. Low levels of E_2 have been implicated in the inhibition of GnRH pulse frequency in rats (Nishihara *et al.* 1994). P_4 enhances E_2-induced negative feedback control of LH release in rats (Goodman 1978). During the oestrous cycle, the pulsatile release of GnRH is present and highly sensitive to the effects of gonadal steroid signalling. In rats, the lowest frequency and amplitude of LH pulses were observed in oestrus phase of the cycle (Gallo 1981).

In males, LH and FSH control the release of testosterone from the Leydig cells of the testes, some of which is aromatized into E_2. P_4 synthesis by the testis is also stimulated by the gonadotrophins. The collective functions of gonadal steroids in males include restraint of GnRH secretion, maintenance of spermatogenesis, thermoregulation and modulation of behaviour.

1.2 The GnRH pulse generator

The development of radioimmunoassays for LH and FSH levels (Midgley 1966, Midgley and Jaffe 1966) enabled the measurement of circulating levels of these hormones. LH is released from the anterior pituitary in a pulsatile fashion and the basal frequency of LH pulses differs between species. LH pulses are observed approximately every 75 minutes (min) in ovariectomised (OVX) monkeys (Dierschke *et al.* 1970) and every 20 min in OVX rats (Gay and Sheth

1972). The discovery of GnRH as the hypothalamic neuropeptide responsible for the release of gonadotrophins (Schally *et al.* 1971b) energised the field to understand the underlying mechanisms. It was suggested that a pulsatile secretion of GnRH is required to induce the pulsatile LH release into the peripheral circulation (Osland *et al.* 1975, Schuiling and Gnodde 1976). This was verified after the development of a radioimmunoassay for GnRH (Nett *et al.* 1973), which enables the detection of the pulses of GnRH through sampling of the portal blood. It was shown that not only is GnRH released in a pulsatile fashion into the portal blood, but that each GnRH pulse is tightly coupled to a pulse of LH (Carmel *et al.* 1976, Clarke and Cummins 1982, Levine and Duffy 1988). Therefore, the pulsatile release of GnRH determines the episodic LH pulses. The term 'GnRH pulse generator' has been used to describe the neural construct responsible for this rhythmic discharge of GnRH since the 1970s (Dierschke *et al.* 1970), even though the exact neural structure of this construct is still unknown.

The GnRH pulse generator is thought to be located in the mediobasal hypothalamus which includes the arcuate nucleus (ARC). Indeed, lesions of the ARC in OVX rats (Blake and Sawyer 1974, Soper and Weick 1980) have been shown to abolish pulsatile LH release. Furthermore, synchronised bursts, or 'volleys', of multiunit electrical activity (MUA), were shown to coincide invariably with LH pulses in OVX ewes (Thiery and Pelletier 1981), rats (Kawakami *et al.* 1982), monkeys (Wilson *et al.* 1984) and goats (Mori *et al.*

1991). Since a large population of GnRH neurones reside in the ARC of monkeys (Silverman *et al.* 1982), it was postulated that the GnRH neurones might possess this inherent rhythmicity. Indeed, cultured GnRH cells are capable of releasing GnRH at a comparable frequency to that observed in both rats and monkeys *in vivo* (Krsmanovic *et al.* 1992, Martinez de la Escalera *et al.* 1992, Wetsel *et al.* 1992, Terasawa *et al.* 1999a). This process was coupled with spontaneous synchronized oscillations of intracellular calcium concentrations and resultant firing of action potentials (Terasawa *et al.* 1999b, Nunez *et al.* 2000, Richter *et al.* 2002). Rhythmic calcium current synchrony was also observed in non-neuronal cells between GnRH neurones (Richter *et al.* 2002), which might explain how GnRH neurones are able to synchronize their activity despite their diffuse distribution in the mammalian brains (King *et al.* 1982). However, GnRH neurones were not found to be located in the ARC of rats (King *et al.* 1982), yet abrupt increase of MUA, reflecting the activity of the GnRH pulse generator, are recorded from this region (Kawakami *et al.* 1982, Kimura *et al.* 1991, Nishihara *et al.* 1991, Goubillon *et al.* 1995, McGarvey *et al.* 2001, Kinsey-Jones *et al.* 2008). Furthermore, the frequency of spontaneous calcium oscillations in cultured mouse GnRH neurones was inconsistent with that of GnRH pulses in the mouse (Jasoni *et al.* 2007). Normal pulsatile LH release persists in OVX rats with mediobasal hypothalamus (no GnRH neurones reside within this region) isolated from the rest of brain (Blake and Sawyer 1974, Ohkura *et al.* 1991). This indicates that the neural construct that constitutes the

rodent GnRH pulse generator does not necessarily include the GnRH neurones. In line with this, recent *in vivo* studies using optogenetic approaches have shown that synchronizing GnRH neurones of mice to fire together in their normal bursting pattern fails to generate LH pulses (Campos and Herbison 2014). In contrast, optogenetic activation of GnRH neurones at the GnRH neuron cell bodies or their terminal projections at ME can induce LH pulses (Campos and Herbison 2014).

1.3 The KNDy signalling systems

Recent studies have identified the neuropeptide, kisspeptin (Kiss1), and Kiss1 receptor (Kiss1r; previously known as G protein-coupled receptor 54, GPR54), as a major regulator of the HPG axis. Kiss1 is encoded by *Kiss1* gene and cleaved into different forms including 54, 14, 13 and 10 (Kp-10) amino acid peptides (Harms *et al.* 2003). All Kiss1 proteins and truncated forms contain a C-terminal 10 amino acid motif which is highly conserved across species (Stafford *et al.* 2002, Terao *et al.* 2004) and has the same affinity and efficacy on the rat receptor (Kotani *et al.* 2001). Unless specifically mentioned, all Kiss1 fragments will be referred to as Kiss1.

Kiss1 was initially described as a suppressor of metastasis in malignant melanoma (Lee *et al.* 1996). In 2003, inactivating mutations of gene *KiSS1r* were found to cause hypogonadotrophic hypogonadism in humans associated

with the delay or absence of puberty onset (de Roux *et al.* 2003, Seminara *et al.* 2003). In addition, humans with activating mutations of gene *KiSS1r* experienced precocious puberty (Teles *et al.* 2008). Animal studies have further substantiated the above facts with Kiss1 or Kiss1r knockout mice showing low levels of gonadotrophins and delayed puberty onset (Funes *et al.* 2003, Seminara *et al.* 2003, d'Anglemont de Tassigny *et al.* 2007, Lapatto *et al.* 2007).

Since the discovery of *KiSS1r* mutations as a cause of hypogonadotrophic hypogonadism, considerable evidence has been generated to show the stimulatory effects of Kiss1 on the gonadotrophins. It has been widely reported that central or peripheral administration of Kiss1 induces an increase in LH and FSH release in rats (Irwig *et al.* 2004, Matsui *et al.* 2004, Navarro *et al.* 2004, Thompson *et al.* 2004, Navarro *et al.* 2005a, Navarro *et al.* 2005b, Patterson *et al.* 2006, Tovar *et al.* 2006), mice (Gottsch *et al.* 2004, Messager *et al.* 2005), sheep (Messager *et al.* 2005, Arreguin-Arevalo *et al.* 2007), monkeys (Shahab *et al.* 2005, Plant *et al.* 2006, Seminara *et al.* 2006) and humans (Dhillo *et al.* 2005, Dhillo *et al.* 2007). Furthermore, Kiss1 has a more potent stimulatory effect on LH release than with FSH release (Navarro *et al.* 2005a, Dhillo *et al.* 2007).

Kiss1-induced gonadotrophin release can be blocked by GnRH antagonists in many species, including rats (Irwig *et al.* 2004), mice (Messager *et al.* 2005) and monkeys (Shahab *et al.* 2005). Both central and peripheral administration of Kiss1 induces c-Fos expression (marker of neuronal activation) in GnRH neurones (Irwig *et al.* 2004, Matsui *et al.* 2004). *In vitro* studies have shown that

Kiss1 can induce a potent long-lasting depolarization in mouse GnRH neurones (Han *et al.* 2005, Liu *et al.* 2008). Therefore, Kiss1 appears to stimulate the HPG axis via activation of GnRH neurones directly. Indeed, expression of *Kiss1r* mRNA has been found in the majority of GnRH neurones in rats (Irwig *et al.* 2004), mice (Han *et al.* 2005, Messager *et al.* 2005) and monkeys (Shahab *et al.* 2005). Specific knockout of Kiss1r in GnRH neurones nullifies the stimulatory effect of Kiss1 on the electrical activity of GnRH neurones (Kirilov *et al.* 2013). In addition, Kiss1 neurones project to GnRH perikarya (Clarkson and Herbison 2006b, Yeo and Herbison 2011, Yip *et al.* 2015) and axon terminals (Decourt *et al.* 2008, Ramaswamy *et al.* 2008, Yip *et al.* 2015), providing anatomical evidence of direct innervations of GnRH neurones by Kiss1 neurones.

Kiss1 and *Kiss1r* are widely expressed throughout the brain, particularly in the hypothalamus. Two principal populations of Kiss1 neurones are located in anteroventral periventricular area (AVPV) and ARC in rodents (Clarkson *et al.* 2009, Oakley *et al.* 2009). In the ARC, Kiss1 neurones co-express dynophin (Dyn) and neurokinin B (NKB). In 2009, patients with severe congenital gonadotrophin deficiency and pubertal failure were found to have inactivating mutations in *TAC3* or *TACR3*, the genes encoding NKB or its receptor neurokinin 3 receptor (NK3R), respectively (Topaloglu *et al.* 2009). It revealed the key role of NKB in the central control of reproduction. However, pharmacological studies investigating the effect of NKB on gonadotrophin release have yielded conflicting results, with some showing stimulatory effects

(Billings *et al.* 2010, Ramaswamy *et al.* 2010, Navarro *et al.* 2011a) and others showing inhibitory effects (Sandoval-Guzman and Rance 2004, Navarro *et al.* 2009a, Kinsey-Jones *et al.* 2012). Similarly, recent animal studies that generated *Tacr3* knockout mice (in rodents, NKB and NK3R were encoded by *Tac2* and *Tacr3*, respectively) or administered antagonists to NK3R failed to recapitulate the symptoms presented in human patients caused by inactivating genetic mutations (Gill *et al.* 2012, Navarro *et al.* 2012, Yang *et al.* 2012). Compared with Kiss1, NKB appears to be a less potent driving force in the HPG axis. Indeed, it has been proposed that the stimulatory effect of NKB on the HPG axis is dependent on Kiss1 signalling (Navarro 2013).

Dyn is an endogenous opioid peptide that preferentially signals through the κ-opioid receptor (Wuster *et al.* 1980). Dyn is involved in the suppression of LH release (Kinoshita *et al.* 1982, Navarro *et al.* 2009a). Furthermore, this inhibitory effect appears to be modulated through the GnRH pulse generator, since central administration (intracerebroventricular, icv) of Dyn antagonist increased LH pulse frequency in rats (Gallo 1990) and reduced the interval between MUA volleys in the ARC of goat (Wakabayashi *et al.* 2010). Indeed, intra-ARC administration of an agonist of the κ-opioid receptor decreased LH pulse frequency in OVX rats (Grachev *et al.* 2012a), indicating that ARC Dyn signalling is inhibitory to the GnRH pulse generator.

Across species, Kiss1 neurones in the ARC have been found to be co-expressed with NKB and Dyn, and are therefore named the Kiss1/NKB/Dyn (KNDy)

neurones (Lehman *et al.* 2010). Furthermore, KNDy neurones in the ARC co-express NK3R and project to one another forming an interconnected network (Krajewski *et al.* 2010). It has been speculated that NKB exerts stimulatory effects on KNDy neurones via NK3R to evoke synchronized bursting activities among KNDy neurones. The resultant release of Dyn extinguishes these bursting activities and imposes a prolonged inhibitory effect on KNDy neurones, until the next episodic oscillation. Each NKB/Dyn oscillation could induce a Kiss1 pulse which activates the GnRH neurones to produce the pulsatile mode of GnRH secretion into the pituitary portal blood. Therefore, the KNDy neurones could represent at least part of the neuronal construct of the GnRH pulse generator (Okamura *et al.* 2013).

1.4 Steroid hormone feedback to KNDy signalling systems

Prior to the discovery of Kiss1 as a driving force in the HPG axis, the mechanisms by which gonadal steroids exert their feedback control on GnRH release were unknown. GnRH neurones *per se* do not express the androgen receptor, oestrogen receptor α (ERα) or P_4 receptor (Shivers *et al.* 1983, Skinner *et al.* 2001). It has been shown that Kiss1 neurones express androgen receptor, ERα and ERβ which indicates that Kiss1 neurones may regulate steroids feedback control on GnRH release (Smith *et al.* 2005a, Smith *et al.* 2005b, Kim *et al.* 2011). Furthermore, different populations of Kiss1 neurones are under

different control by gonadal steroids. In both male and female mice, expression of *Kiss1* mRNA in the ARC is increased by gonadectomy, but reduced by the replacement of gonadal steroids (Smith *et al.* 2005a, Smith *et al.* 2005b). Conversely, treatment with gonadal steroids increases the expression of *Kiss1* mRNA in the AVPV in both male and female mice (Smith *et al.* 2005a, Smith *et al.* 2005b). Similarly, in both male and female rodents, gonadal steroids increase the expression of *Kiss1* mRNA in the medial amygdala (MeA), a region implicated in social and emotional behaviours as well as various aspects of reproduction (Kim *et al.* 2011). Although the physiological relevance of Kiss1 neurones in the MeA remains unknown, there is abundant evidence to suggest that AVPV and ARC Kiss1 neurones are involved in distinct attributes of gonadal steroids feedback control of GnRH release.

In female rodents, AVPV Kiss1 neurones have been proposed to relay the positive feedback effects of E_2 on GnRH neurones during the generation of the preovulatory LH surge. The expression of *Kiss1* mRNA in the AVPV in females peaked during the afternoon of the proestrus stage, and the AVPV Kiss1 neurones were strongly activated during the LH surge, measured by the c-Fos expression in these neurones (Smith *et al.* 2006, Adachi *et al.* 2007, Clarkson *et al.* 2008). Central administration of Kiss1 antagonist into the mPOA where GnRH neurones reside blocked the spontaneous and E_2-induced LH surges in female rats (Kinoshita *et al.* 2005, Adachi *et al.* 2007). Similarly, chronic administration (icv) of specific Kiss1r antagonist abolished the spontaneous LH

surges (Pineda *et al.* 2010a). *Kiss1* or *Kiss1r* knockout female mice also failed to generate preovulatory LH surges, accompanied with decreased GnRH neuronal activities during that time (Clarkson *et al.* 2008). Furthermore, ERα knockdown in Kiss1 neurones blocked ovulation in female mice, substantiating the hypothesis that the positive feedback control of E_2 on GnRH secretion are mediated by Kiss1 neurones in the AVPV through ERα signalling (Mayer *et al.* 2010).

Contrary to Kiss1 neurones in the AVPV, ARC Kiss1 neurones regulate the negative feedback control of gonadal steroids. The up-regulating of *Kiss1* mRNA expression in the ARC following OVX corresponded with an increase in circulating levels of gonadotrophins (Smith *et al.* 2005a). This increase was significantly attenuated in female mice lacking ERα in Kiss1 neurones (Mayer *et al.* 2010). In the ARC, Kiss1 neurones co-express NKB and Dyn. Like Kiss1, NKB and Dyn are both involved in the negative feedback control of gonadal steroids. The expression of *Tac2* mRNA in the ARC was elevated after OVX and reduced after E_2 treatment in female rats (Dellovade and Merchenthaler 2004). The reduction in *Tac2* mRNA levels by E_2 was nullified in ERα knockout female mice (Dellovade and Merchenthaler 2004). This indicates that ERα signalling is also involved in NKB modulated E_2 negative feedback control. Dyn has been involved in P_4-induced inhibition of pulsatile GnRH release. Dyn neurones in the ARC express receptors for P_4 (Foradori *et al.* 2002) and central administration of Dyn antagonist blocked P_4-induced inhibition of pulsatile LH

release in sheep (Goodman *et al.* 2004). As expected, gonadectomy decreased the *Dyn* mRNA expression in the ovine ARC (Foradori *et al.* 2005). However, the expression of *Dyn* mRNA in the ARC remained unchanged by P_4 treatment or inhibited by E_2 treatment (Foradori *et al.* 2005, Gottsch *et al.* 2009, Navarro *et al.* 2009a), despite its suppressive effects on LH release. Nevertheless, the ERα signalling seems to be involved in the E_2 negative feedback control by Dyn, since the inhibition of *Dyn* mRNA by E_2 treatment was abolished in female mice lacking ERα (Gottsch *et al.* 2009). In summary, KNDy neurones in the ARC are the principal mediators regulating gonadal steroids-induced negative feedback control of the gonadotrophins.

1.5 KNDy signalling in reproductive development

Puberty is defined as acceleration of growth, achievement of reproductive competence and development of secondary sexual characteristics (Grumbach 2002). The vaginal opening, an external marker of puberty onset, usually occurs a day after the first ovulation in most laboratory rats (35-45 d after birth) (Heger and Ojeda 2007). In female rhesus monkeys, menarche and first ovulation occurs at approximately 30 and 42 month of age, respectively (Plant 2007). Although rodent puberty is different from the the monkeys due to an absent juvenile brake on the pulsatile GnRH release, the basic mechanisms underlying the process of sexual maturation are well conserved across species. The nature of puberty is an

activation of the HPG axis with an accelearated pulsatile release of GnRH (Wildt *et al.* 1980, Urbanski and Ojeda 1985), which in turn increases the circulating levels of the gonadotrophins and gonadal steroids. It has been shown that both the frequency and amplitude of GnRH pulses increases peripubertally in both rats and monkeys (Watanabe and Terasawa 1989, Sisk *et al.* 2001, Harris and Levine 2003). Furthermore, a reduction in the sensitivity of the E_2-induced negative feedback control of gonadotrophin release during the peripubertal period might facilitate this activation of the HPG axis (Bronson 1981, Rapisarda *et al.* 1983). Although the exact mechanisms underlying the initiation of puberty are still unknown, many studies have implicated the KNDy signalling systems in the acceleration of GnRH release during puberty onset.

During the peripubertal period, expression of *Kiss1* mRNA in the ARC increases significantly in both male and female rats (Takase *et al.* 2009, Bentsen *et al.* 2010). Chronic administration (icv) of Kiss1 advances the onset of puberty (Shahab *et al.* 2005, Clarkson and Herbison 2006b), whereas Kiss1r antagonism results in pubertal delay in rats (Pineda *et al.* 2010a). Furthermore, inactivating mutations of Kiss1 impairs reproductive maturation in both male and female mice (d'Anglemont de Tassigny *et al.* 2007). Likewise, levels of ARC *Tac2* mRNA expression increase gradually across sexual maturation and peak at puberty onset (Gill *et al.* 2012, Navarro *et al.* 2012). It has been postulated that KNDy neurones in the ARC play an important role in puberty onset simply because KNDy neurones compose part of neural construct of the GnRH pulse

generator (Terasawa *et al.* 2013). Indeed, the frequency and amplitude of Kiss1 pulses measured at the level of the ME are gradually increased in prepubertal monkeys (Keen *et al.* 2008, Guerriero *et al.* 2012), in parallel to the prepubertal developmental changes in GnRH release. This increase of Kiss1 release is accompanied by an increasing sensitivity of GnRH neurones in response to Kiss1 (Castellano *et al.* 2006, Roa *et al.* 2008).

It should be noted that E_2-induced negative feedback control of ARC Kiss1 signalling exists before the puberty onset. Treatment with E_2 decreased the ARC *Kiss1* and *Tac2* mRNA in prepubertal mice (Gill *et al.* 2012). Furthermore, targeted knockdown of ERα expression in Kiss1 neurones increased ARC *Kiss1* mRNA and advanced puberty onset in mice (Mayer *et al.* 2010). However, both ARC *Kiss1* and *Tac2* mRNA expression increased gradually during sexual maturation, despite the increased circulating E_2 levels during that period (Takase *et al.* 2009, Gill *et al.* 2012). The treatment with E_2 in prepubertal monkeys did not inhibit the pulsatile release of Kiss1 at the level of the ME (Guerriero *et al.* 2012). Therefore, the increased activity of ARC Kiss1 and NKB signalling seem to override the negative feedback control of the gonadal steroids peripubertally. However, the underlying mechanisms for up-regulated KNDy signalling peripubertally are still unknown.

The pubertal activation of the HPG axis is influenced by metabolic signals. The timing of puberty onset is closely related to body weight (Kennedy and Mitra 1963). Negative energy balance delays puberty onset, whilst over-nutrition

advances puberty onset. The KNDy signalling systems have been implicated in mediating the effects of metabolic signals on the developing HPG axis. Fasting induced a decrease of hypothalamic *Kiss1* mRNA expression in prepubertal rats, with significantly delayed puberty onset (Castellano *et al.* 2005). Likewise, both ARC *Tac2* and *Tacr3* mRNA expression were down-regulated in prepubertal fasting rats (Navarro *et al.* 2012). Treatment of Kiss1 or NKB during the fasting period could restore the normal gonadotrophin release and puberty onset (Castellano *et al.* 2005, Navarro *et al.* 2012). In contrast, over-nutrition-induced precocious puberty in rats is related to the increased pulsatile LH release and up-regulated ARC *Kiss1* and *Tac2* expression (Li *et al.* 2012). This indicates that metabolic cues affect the timing of puberty onset by mediating the KNDy signalling systems.

1.6 Neuropeptides regulate the KNDy signalling systems

Kiss1 belongs to a peptide family named RFamide peptides which possess an Arg-Phe-NH_2 motif at their C-terminus. RFamide peptides have recently been characterised as major regulators of GnRH neurones (Parhar *et al.* 2012). Among them, gonadotrophin-inhibitory hormone (GnIH) and its mammalian orthologue RFamide related peptides (RFRP) are emerging as important inhibitory neuropeptides on the HPG axis.

In 2000, it was discovered that GnIH is able to directly inhibit gonadotrophin

secretion at the level of the anterior pituitary gland in the quail (Tsutsui *et al.* 2000). In avian species, GnIH neurones were found in the paraventricular nucleus (PVN), with fibres projecting to the ME (Tsutsui *et al.* 2000, Ukena *et al.* 2003). The receptor for GnIH exists in the avian pituitary and GnIH inhibits gonadal development and maintenance via the decrease of gonadotrophin synthesis and release in the pituitary (Ubuka *et al.* 2006). In mammals, the orthologous GnIH gene encodes a precursor polypeptide, the RFRP (Hinuma *et al.* 2000, Ukena *et al.* 2002). The expression of *RFRP* mRNA was detected in the dorsomedial hypothalamic area (DMH) in rodents (Kriegsfeld *et al.* 2006). The majority of *RFRP* mRNA expressing neurones were localised in the periventricular nucleus (IPe) in monkeys (Ubuka *et al.* 2009), and in the DMH and PVN in sheep (Clarke *et al.* 2008). In mammals, the projections of RFRP neurones were traced to multiple limbic and hypothalamic structures including the mPOA, ARC and amygdala (Kriegsfeld *et al.* 2006, Johnson *et al.* 2007, Ubuka *et al.* 2009, Ubuka *et al.* 2012). Across species, RFRP fibres were observed to innervate the GnRH neurone cell bodies in the mPOA (Kriegsfeld *et al.* 2006, Smith *et al.* 2008). This indicates that RFRP may exert a direct inhibitory effect on the GnRH neurones. Indeed, G protein-coupled receptor 147 (GPR147), the receptor of RFRP, is expressed in GnRH neurones (Ubuka *et al.* 2012) and central administration of RFamide related peptide-3 (RFRP-3) has been shown to decrease GnRH neuronal activities and induce an inhibition of gonadotrophin release (Kriegsfeld *et al.* 2006, Johnson *et al.* 2007, Anderson *et*

al. 2009, Pineda *et al.* 2010b). In addition, RFRP-3 decreased the firing rate of GnRH neurones *in vitro* (Ducret *et al.* 2009). Furthermore, species differences may exist with regard to the fibre projections of RFRP in the ME. RFRP fibres were observed in the external (neurosecretory) layer of ME in sheep, while no obvious RFRP fibres were found in the ME of rodents (Kriegsfeld *et al.* 2006, Johnson *et al.* 2007, Clarke *et al.* 2008). Furthermore, RFRP neurones failed to take up retrograde tract tracing dye injected peripherally in rodents (Rizwan *et al.* 2009). Therefore, RFRP predominately regulates the HPG axis via central mechanisms in rodents. The recent findings that ARC Kiss1 neurones express GPR147 and receive projections from RFRP neurones (Poling *et al.* 2013) indicate that the KNDy signalling systems might be involved in this process.

Like the KNDy signalling systems, RFRP has been involved in gonadal steroids feedback control of gonadotrophins. RFRP neurones express ERα receptors and peripheral treatment of E_2 suppressed the hypothalamic *RFRP* mRNA in mice (Kriegsfeld *et al.* 2006, Poling *et al.* 2012). Furthermore, OVX induced a higher LH level in *GPR147* knockout mice compared to wild type mice (Leon *et al.* 2014). This indicates that RFRP could be involved in the E_2-induced negative feedback control. However, this might not be the case in rats since OVX did not affect the expression of *RFRP* mRNA in DMH in rats (Quennell *et al.* 2010). RFRP has also been implicated in E_2 positive feedback control of gonadotrophin release. RFRP-3 neuronal activities were reduced during the LH surge in hamsters (Gibson *et al.* 2008). Furthermore, central administration of RFRP-3

inhibited neuronal activities in AVPV and blocked the E_2-induced LH surge in female rats (Anderson *et al.* 2009). However, normal pre-ovulatory LH surges were observed in *GPR147* knockout mice, indicating that RFRP-3 may just play a permissive role in the positive feedback control (Leon *et al.* 2014). In summary, the gonadal feedback control of RFRP signalling is still not clear and merits further investigations.

NKB belongs to a family of peptides termed tachykinins, which play an essential role in the regulation of HPG axis (Lasaga and Debeljuk 2011). The best known members of this family are substance P, neurokinin A (NKA) and NKB, which preferentially bind to three G protein-coupled receptors NK1R, NK2R and NK3R respectively (Almeida *et al.* 2004). Substance P can influence the HPG axis via acting at the level of hypothalamus, pituitary and gonads (Lasaga and Debeljuk 2011). There is considerable evidence that has implicated substance P in regulating the release of gonadotrophins and prolactin (Vijayan and McCann 1979, Dees *et al.* 1985, Picanco-Diniz *et al.* 1990), although with conflicting results of either inhibitory or stimulatory effects reported. Furthermore, substance P has been shown to suppress LH surges in rats (Aslanowicz-Antkowiak and Traczyk 1992) and monkeys (Kerdelhue *et al.* 1997). However, with the exception of NKB, research on the role of tachykinins on HPG axis in the last decade has been rather modest (Lasaga and Debeljuk 2011). Recent studies have energised this field by showing that substance P is co-expressed with Kiss1 and NKB in KNDy neurones in the human

(Hrabovszky *et al.* 2013). This indicates that substance P might be a fourth player in the existing KNDy signalling systems. However, this was not replicated in mice (Navarro *et al.* 2015). Furthermore, substance P directly increases the excitability of mouse ARC Kiss1 neurones, which express NK1R (de Croft *et al.* 2013, Navarro *et al.* 2015). The stimulatory effect of substance P on LH release is abolished in *Kiss1r* knockout mice (Navarro *et al.* 2015). In summary, the role of substance P in central regulation of HPG axis involves the KNDy signalling systems.

Glutamate and gamma-aminobutyric acid (GABA) are two principal neurotransmitters (stimulatory and inhibitory, respectively) of the central nervous system. Glutamate, acting through the N-methyl-D-aspartate (NMDA) receptor, and GABA, through $GABA_A$ and $GABA_B$ receptors, play an important role in many aspects of the reproductive system, including the onset of puberty, pre-ovulatory LH surges, and the pulsatile release of LH (Jarry *et al.* 1991, Meijs-Roelofs *et al.* 1991, Ping *et al.* 1995, Clarkson and Herbison 2006a). Although both glutamate and GABA appear to modulate GnRH neurones directly, since GnRH neurones express NMDA, $GABA_A$ and $GABA_B$ receptors (Jung *et al.* 1998, Adjan *et al.* 2008, Liu and Herbison 2011), recent studies have provide evidence to suggest that glutamate and GABA could regulate GnRH neurones indirectly through the Kiss1 signalling system. Fasting-induced decreased expression of *Kiss1* mRNA in monkeys is associated with a reduction in glutamatergic inputs to Kiss1 neurones via NMDA receptors (Shamas *et al.*

2015). Furthermore, peripherally administered NMDA-induced LH secretion in mice is dependent on Kiss1 signalling (d'Anglemont de Tassigny *et al.* 2010). Likewise, antagonism of $GABA_A$ receptor in the ME/ARC in monkeys increased the release of Kiss1 and resultant GnRH, which can be blocked by a Kiss1r antagonist (Kurian *et al.* 2012). Similarly, knockout of $GABA_B$ receptor amplified the expression of *Kiss1* mRNA in the MeA in mice with increased release of GnRH (Catalano *et al.* 2010, Di Giorgio *et al.* 2014). Taken together, Kiss1 signalling may function as a downstream effector of glutamatergic and GABAergic signalling on the GnRH neurones.

1.7 Aims and objectives

The discovery of KNDy and other related neuropeptide signalling systems have rapidly advanced our knowledge with regard to the central control of the HPG axis. However, ambiguous or even contradictory evidence has been produced regarding their roles in the control of puberty onset and gonadotrophin release. Furthermore, even less is known about their roles in regulating the pulsatile release of GnRH. Therefore, the general aim of the studies outlined within this thesis is to provide experimental evidence to address these questions. More specific objectives are listed below.

1. To investigate the role of endogenous NKB-NK3R signalling in regulating the

puberty onset.

- To determine the effects of central administered NK3R antagonist on the timing of puberty onset in female rats and whether it could affect the precocious puberty onset induced by over-nutrition status.

- To test the hypothesis that NKB-NK3R signalling alters the dynamic of pulsatile GnRH release in prepubertal female rats to affect the timing of puberty onset.

2. To interrogate the action of ovarian steroid hormone on gonadotrophin release in response to NKB-NK3R and NMDA signallings.

- To determine the effects of NK3R agonist or NMDA on LH release in OVX rats replaced with different levels of E_2.

- To establish whether the biphasic effects of NMDA on LH release in different E_2 conditions are mediated through the KNDy signalling systems.

3. To investigate the role of the NK1R signalling system in the ARC in the regulation of gonadotrophin release.

- To test the effects of NK1R signalling in the ARC on pulsatile LH release in OVX rats.

- To determine the modulatory influence of E_2 on LH release in OVX rats administered with NK1R agonists into the ARC.

4. To elucidate the role of RFRP-3 signalling in the central control of the HPG axis.

- To determine whether RFRP-3 signalling interacts with endogenous opioid peptides to regulate GnRH pulse generator activity.

- To examine the role of RFRP-3 signalling in stress-induced suppression of GnRH pulse generator frequency.

- To determine the mechanism and site of action of RFRP-3 in the E_2-induced negative feedback control of LH release.

5. To investigate the role of Kiss1 and GABA signallings in the MeA in regulating LH secretion.

- To determine the effects of intra-MeA administration of Kiss1 and Kiss1r antagonists on pulsatile LH secretion in OVX rats.
- To examine the effects of chronic administration of Kiss1r antagonists on spontaneous LH surges and oestrous cyclicity in female rats.

- To determine whether the stimulatory effects of peripherally administered Kiss1 on LH secretion is mediated by $GABA_B$ receptor signalling in the MeA in OVX rats.

CHAPTER TWO: GENERAL MATERIALS AND METHODS

2.1 Experiments on prepubertal animals

2.1.1 Animals

Adult Sprague-Dawley rats obtained from Charles River (Margate, UK) were allocated into breeding pairs. Once the female rats were suspected of pregnancy, they were separated from the male rat into an individual cage and fed with breeding diet (12% kcal fat, 27% kcal protein, 61% kcal carbohydrate; RM3; Special Diet Services). Litters were assessed every day (d) and the d of birth was considered postnatal d (pnd) 0. Excess male offspring were culled to reduce litters to 10-12 pups on pnd 2. Litters were weaned at pnd 21 and female offspring were housed in groups of four or six per cage under controlled conditions (12-h light, 12-h darkness, with lights on at 0700 h; controlled ambient temperature 22 ± 2 °C) and provided with food and water *ad libitum*. Litters were either assigned to standard chow diet (21% kcal fat, 17% kcal protein, 63% kcal carbohydrate; RM1; Special Diet Services) or a high fat diet (45% kcal fat, 20% kcal protein, 35% kcal carbohydrate; Special Diet Service). Litters were weighed every 3-d after pnd 25 and monitored daily for vaginal opening and first oestrous after pnd 28. All procedures were conducted in accordance with Animals (Scientific Procedures) Act UK, 1986 and were approved by the King's College London Ethical Review Panel Committee.

2.1.2 Surgical procedures

2.1.2.1 Anaesthesia and post-operative care

All surgical procedures were carried out under a combination of ketamine (Vetalar, 600 mg/kg, ip; Pfizer, Sandwich, UK) and xylazine (Rompun, 60 mg/kg, ip; Bayer, Newbury, UK); supplementary injections of ketamine (600 mg/kg, ip) were administered to maintain anaesthesia as required. Each rat was injected subcutaneously with 0.8 ml/kg Duphamox LA antibiotic suspension (200 mg/ml procaine benzylpenicillin, 250 mg/ml dihydrostreptomycin-sulphate; Fort Dodge Animal Health, Southampton, UK) prior to surgery. Following each surgical procedure, animals were allowed to recover from anaesthesia on a heated pad until fully conscious. Post-operative analgesia was provided by means of subcutaneous administration of carprofen (Rimadyl; 4.4 mg/kg; Pfizer Animal Health, Tadworth, UK) daily for 3 d.

2.1.2.2 Head screws for tether attachment

In order to allow the anchorage of a metal spring to protect exteriorised chronically implanted intravenous (iv) catheters used for blood sampling, the animals were fitted with a tether screw on the skull. Under general anaesthesia, the scalp of the animal was shaved and the head was then placed in a stereotaxic frame (David Kopf Instruments, Tujunga, California, USA) by placing the ear bars into the external auditory meatus on each side of the skull, which was then

balanced and tightened. The incisors were placed over the incisor bar (ensuring the tongue clearance) and the nose was held firmly down with the nose bar. The skin of the scalp was sterilized by swabbing with povidoneiodine surgical scrub (Betadine; Seton Healthcare, Oldham, UK) followed by 75% ethanol. The eyes were protected by *ad hoc* topical application of ophthalmic gel (2mg/g Carbomer 980; Viscotears; Novartis, Camberley, UK) and an opaque sterile shield. A midline incision (approximately 1.5 cm) was made with a scalpel and the skin was retracted. The periosteum was removed using a scalpel blade and the skull was dried with sterile gauze.

Three screw holes, two in the occipital bone and a third on the right hand side of the frontal bone, were drilled with a dental bur (RdPC3, Ash® Instruments; Dentsply, Addlestone, UK) in a handheld cordless drill (Interfocus, Linton, UK). Three stainless steel cheese head screws (10BA × 1/32”; Allscrews, Reading, UK) were then screwed into the holes. The screws provide anchorage over smooth surface of the skull. The slotted screw was held inverted in a micro-manipulator (David Kopf Instruments) on the stereotaxic frame, and was then secured in a midline position just rostral to the two cheese head screws in the occipital bone. Dental cement, which was made from Simplex rapid acrylic powder (Associated Dental Products, Swindon, UK) containing streptomycin antibiotic (1:14; Sigma-Aldrich) and mixed with solvent (Associated Dental Products), was applied to the three small screws and the head of the tether screw until all screw heads were completely covered. Once the dental cement had dried,

the incision was closed around the shaft of the slotted screw using 5.0 mercerised silk (Pearsalls, Taunton, Somerset, UK). The animal was then removed from the stereotaxic frame, and was allowed to recover on a heat pad. Once recovered, the animals were returned to their individual cages. All experimental surgical procedures were carried out with the aid of a surgical microscope (Carl Zeiss, Welwyn Garden City, UK) and a fibre-optic cold light source (KL 1500 LCD; Schott, Stafford, UK) if required.

2.1.2.3 Unilateral cannulation of the internal jugular veins

On pnd 31 (fed with normal chow diet) or pnd 29 (fed with high fat diet), the animals were implanted with iv catheters for collection of blood samples. The iv catheters were constructed from 42 cm lengths of polypropylene tubing (Portex PP50; Smiths Medical, Ashford, UK) inserted, allowing 15 mm overlap, into 4 cm lengths of fine Silastic™ medical grade tubing (STHT-C-020-0, Sanitech) at the proximal end and, with a 10 mm overlap, into 5 cm lengths of size 0.02 Tygon tubing at the distal end, each transiently expanded immersion in xylene. A 20 cm length of 5.0 mercerised silk was then tied in the middle of the overlap between the PP50 and Silastic™ tubing and was held in place using a ring of silicone adhesive, such that the two ends of the silk ligature were of equal length. The catheters were then left overnight to allow the silicone adhesive to dry. The Silastic™ tubing was cut to length of 19 mm, measured from the ring of silicone

adhesive. The resulting catheters were flushed with sterile saline and a saline-filled 2 ml syringe (Terumo, Egham, UK) was attached to the distal end of the catheter.

The iv catheterisation was carried out under general anaesthesia, as described above. The ventral surface of the animal's neck was shaved using electric hair clippers. The animal was placed on the dorsum on top of heat pad with the neck resting on a plastic support to facilitate catheter implantation. The shaved area was then sterilised with surgical scrub and ethanol as described above. A 1 cm longitudinal incision was made along the midline over the trachea and the skin was retracted with clamps. The right internal jugular vein was then exposed following the method of blunt dissection. Loops of 5.0 mercerised silk were placed around the vessel, proximal and distal to the exposed portion, lifting it slightly to allow ease of access. The proximal suture (but not the distal suture) was then tightened to close the vein and prevent back-flow. A transverse incision encompassing 25% of the vein's circumference was made using Castroviejo micro-dissection scissors (Harvard Apparatus, Edenbridge, UK). The Silastic^{TM} end of the catheter was inserted into the vessel through the incision until the ring of silicone cement reached the incision. At this point the tip of the catheter is positioned at the junction between the cranial (superior) vena cava and the right atrium; this was confirmed by observation of effortless drawback and replacement of blood using the saline-filled syringe. The catheter was secured to the vein by the loops of 5.0 mercerised silk. The 5.0 mercerised silk attached to

the catheter by silicone adhesive was sutured to the pectoralis major muscle to keep the catheter in place. The syringe was replaced with a pin resembling a 10 mm length of stainless steel wire (diameter = 0.65 mm; K.C. Smith, Potters Bar, UK). Crocodile forceps (Harvard Apparatus) were introduced at the posterior portion of the incision proximal to the slotted screw anchored to the animal's cranium, tunnelled under the skin around the neck until their tips were exposed through the ventral incision. The distal end of the jugular catheter on the same side was then held firmly in the crocodile forceps and retracted under the skin until exteriorised through the incision on the head. This was repeated for the second catheter and the neck incision was sutured with 5.0 mercerised silk. The catheters were tunnelled through a 30 cm long protective spring tether (Instec), which was then anchored to the slotted screw. A small nut fixed with thread-locking adhesive (Henkel Loctite Adhesives, Welwyn Garden City, UK) was then used to secure the tether to the cranial attachment. Following surgery the animals were allowed to recover from anaesthesia on a heated pad until fully conscious.

After the animals regained consciousness, catheters were filled with 0.2 ml 40% polyvinyl pyrolidone (Sigma-Aldrich) in heparinised (200 U/ml heparin sodium; CP Pharmaceuticals, Wrexham, UK) saline and 2 mg/ml streptomycin (Sigma-Aldrich). The stainless steel pins were then replaced. The solution acts as a flexible, removable physical barrier that helps to maintain cannula patency, and also has anti-bacterial and anti-fungal properties to improve cannula

longevity and minimise risk of infection. The animals were then returned to their individual cages, and the distal end of the spring tether was attached to a swivel (Instec), which rotates 360° in the horizontal plane, allowing the animals to move freely around the enclosure. The animals were left to recover from surgery for 2-d before experiments commenced.

2.1.3 Collection of blood samples

Blood collecting procedures were carried out on pnd 34 and 36 in rats fed with a standard chow diet, and on pnd 31 and 33 in rats fed with a high fat diet. The computer-controlled automated serial blood sampling system was based on a system described previously (Clark *et al.* 1986). The 8-channel system allows for the intermittent withdrawal of small blood samples (35 µl) from up to 8 rats simultaneously with minimal disturbance to the animals. On the morning of experimentation, the polyvinyl pyrolidone solution was aspirated from the indwelling iv catheter, the cannula was flushed with physiological saline and connected to one of the channels of a dual-channel swivel (Instec). Once connected, the animals were left undisturbed for 2 h. Blood sampling commenced at between 1100 and 1200 h, and samples were collected every 5 min for up 4 h.

A 5 cm length of size 0.02 Tygon tubing connected the distal end of the dual-channel swivel to the first port on a three-way solenoid valve (Lee Products,

Gerrards Cross, UK). A 162 cm length of polypropylene tubing (Portex PP10; Smith's Medical) was attached to the second port of the solenoid valve, with the distal end connected to a modified hypodermic needle (25 gauge; Terumo) attached to a robotic fraction collector (222XL; Gilson, Middleton, WI, USA). Finally, a 176.5 cm length of polypropylene tubing (Portex PP30; Smith's Medical) was used to connect the third port on the solenoid valve with a bag of heparinised (50 U/ml heparin sodium) saline via a length of peristaltic pump tubing (Gilson) mounted on an 8-channel peristaltic pump (Gilson).

Custom-made software (provided by Mr D. Layman, King's College London) controlled the system through a System Interface Module (Gilson) linked to the peristaltic pump, the fraction collector and the solenoid valves. The third port on the solenoid, leading to the saline reservoir, was always open whilst the computer switched between the first port (to the rat) and the second port (to the fraction collector). Figure 2.1 provides a schematic representation of the bleeding system. Each cycle started with the first port on the solenoid valve opening and saline from the reservoir being delivered to the rat for 4 seconds (s) to flush the iv cannula. The solenoid then switched to open the second port, closing the first port towards the animal, and saline was flushed to the fraction collector for 20 s to flush the associated tubing. After that, the valve to the fraction collector was closed, the valve to the rat opened, and the direction of the peristaltic pump was reserved to allow withdrawal of blood from the rat for 65 s. The solenoid valve was switched again to the fraction collector and the pump

direction again reversed. The pump then flushed the blood through the solenoid into the PP10 tubing leading to the fraction collector in 15 s. At the preset pump speed and the given internal diameters of tubing used, the rate of blood withdrawal from each prepubertal animal was 35 µl in 15 s. The second port on the solenoid valve, towards the fraction collector, was then closed and the first port opened for 88 s so that blood remaining within the iv cannulae could be returned to the animal and 35 µl heparinised saline was administered to maintain blood volume. The first port was closed and the second port opened to the fraction collector, and the 35 µl blood held in the PP10 tubing was pumped through to the fraction collector and delivered into one of a series of labelled LP-2 tubes (Thermo Life Sciences, Basingstoke, UK). In addition to the 35 µl of blood, heparinised saline from the reservoir was pumped through the fraction collector. This ensured that all of the blood was delivered into the tubes and that the fraction collector tubing was flushed at the end of each cycle. This final volume within each LP-2 tube was 185 µl (35 µl blood and 150 µl heparinised saline). The system then stood by for 28 s before starting the next cycle, in order to maintain a 5-min interval between each blood withdrawal. A schematic diagram summarising the stages of the automated blood sampling cycle is provided (Figure 2.2).

After the completion of all of the sampling cycles, the iv cannulae were disconnected from the bleeding system, flushed with sterile saline, refilled with 0.2 ml polyvinyl pyrolidone solution and plugged with stainless steel pins to

maintain patency, and the animals were removed from the system. The system was flushed with sterile water followed by 75% ethanol, each for 10 min, in order to maintain patency and reduce the risk of bacterial contamination. The collected blood samples were frozen at -20°C.

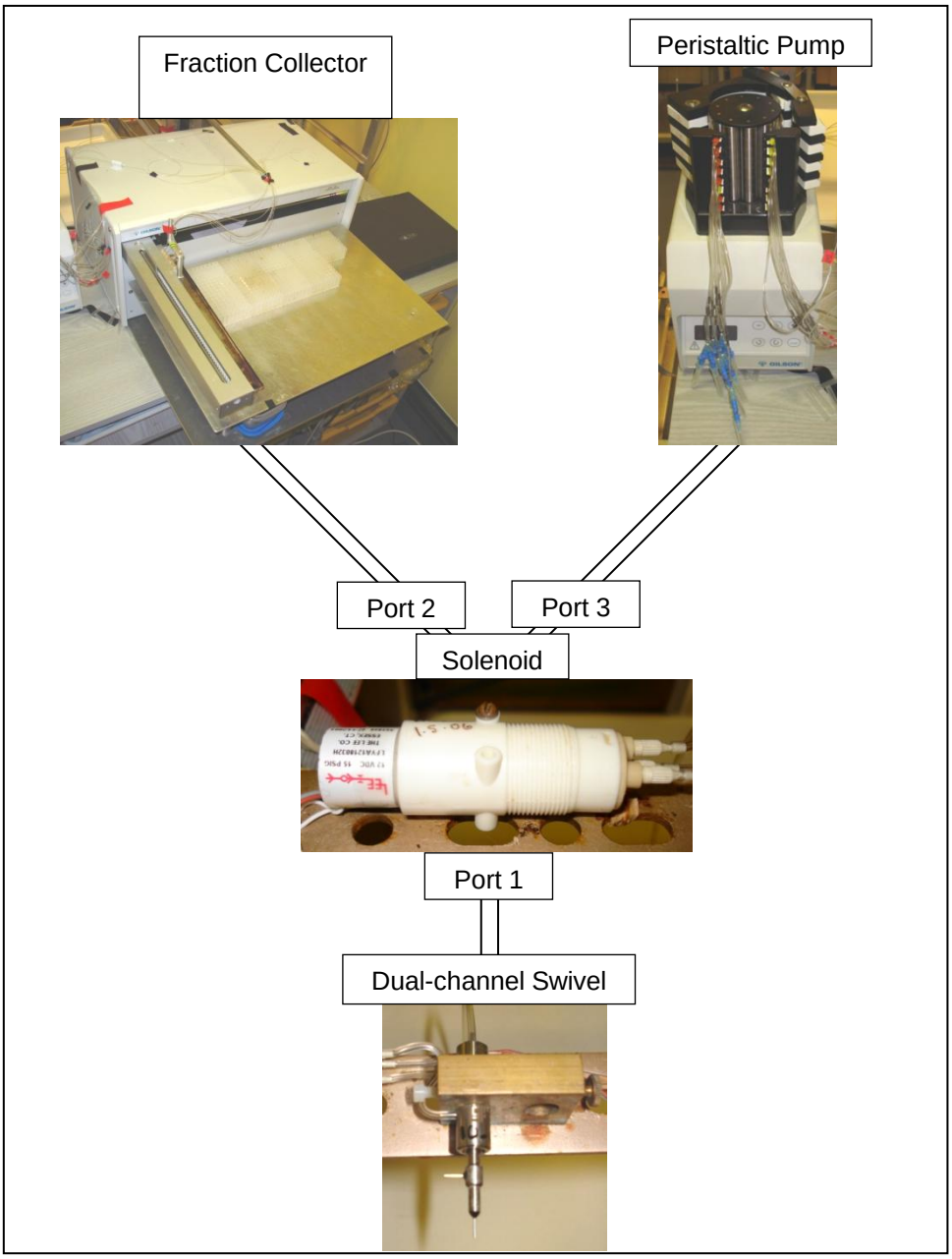

Fig. 2.1 Photographs of the automated blood sampling system components, showing the connections from ports 1, 2 and 3 on the solenoid valve to the dual-channel swivel, fraction collector and peristaltic pump respectively.

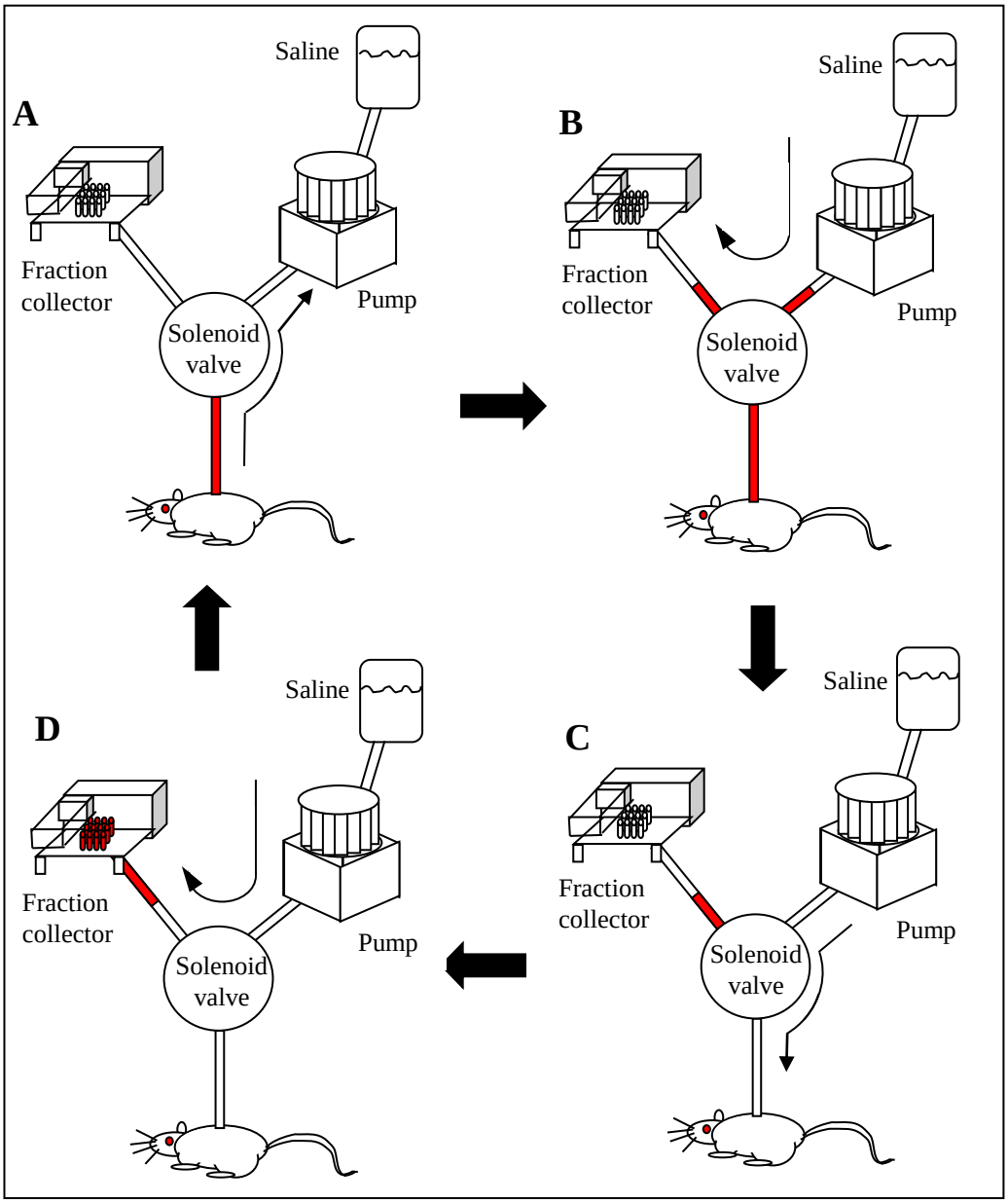

Fig. 2.2 Simplified schematic diagram of the automated blood sampling system cycle. (A), Blood is first withdrawn from the rat towards the pump. (B), 25 µl (or 35 µl) of blood is then pushed from the pump towards the fraction collector. (C), The remaining blood, together with 25 µl (or 35 µl) heparinised saline to maintain blood volume, is then returned to the animal. (D), The 25 µl (or 35 µl) blood sample is deposited via the fraction collector into a sample tube.

2.2 Experiments on adult animals

2.2.1 Animals

Experiments were carried out using adult Sprague-Dawley rats, weighing 200-220 g, obtained from Harlan Laboratories (Oxfordshire, UK). All animals were housed under controlled conditions, as described above, provided with standard chow diet and water *ad libitum*. Animals were group-housed (maximum four per enclosure) prior to surgery and housed individually following surgery and during experimentation. All animal procedures were conducted legally and ethically, as described above.

2.2.2 Surgical procedures

2.2.2.1 Anaesthesia and post-operative care

All surgical procedures were carried out under general anaesthesia induced by ketamine (Vetalar, 100 mg/kg, ip; Pfizer, Sandwich, UK) co-administered with and xylazine (Rompun, 10 mg/kg, ip; Bayer, Newbury, UK); supplementary injections of ketamine (100 mg/kg, ip) were administered to maintain anaesthesia as required. Each rat was injected subcutaneously with 0.4 ml/kg Duphamox LA antibiotic suspension (Fort Dodge Animal Health) prior to surgery. Animals were allowed to recover from anaesthesia on a heat pad after each surgical procedure until fully conscious. Post-operative analgesia was provided by means of subcutaneous administration of 4.4 mg/kg carprofen

(Pfizer Animal Health) daily for 3 d.

2.2.2.2 Ovariectomy

Rats were bilaterally OVX in order to remove fluctuating levels of gonadal steroids. Animals were anaesthetised and an appropriate area in the midline of the back, between the bottom rib and the top of the thigh was shaved with an electric razor. The shaved area was sterilised by Betadine surgical scrub followed by 75% ethanol as described above. An incision (approximately 2 cm in length) was made along the midline of the back, laterally over the estimated position of the ovaries. Scissors were then used to separate the abdominal muscle 1 cm left lateral to the spine, and the ovary was retrieved with blunt forceps. The fallopian tube was then clamped below the full ovary and the distal end of the uterine horn, tied off with a 3.0 mercerised silk suture and the ovary was removed with a scalpel. The remaining fallopian tube and uterus were then replaced in the abdominal cavity and the muscle incision was closed with a 3.0 mercerised silk suture. The procedure was repeated to remove the contralateral ovary. In addition, animals receiving E_2 replacement were implanted subcutaneously with either one or two Silastic^{TM} E_2 capsules according to the experimental design. The E_2 capsules were constructed from 5 cm lengths of Silastic^{TM} tubing (inner diameter [ID] 1.57 mm; outer diameter [OD] 3.18 mm; Sanitech, Havant, UK). The tubing was sealed at one end with implant grade

silicone adhesive (Silibione MED ADH 4100 RTV; Bluestar Silicones, Ventura, CA, USA) and then left to dry overnight. The following d, the tubing was filled to a length of 25 mm with 20 μg/ml E_2 (Sigma-Aldrich) dissolved in arachis oil (Sigma-Aldrich), and the open end of the tubing was again sealed with silicone adhesive. Control animals without E_2 replacement were replaced with two 5 cm lengths of Silastic™ capsules filled with arachis oil (Sigma-Aldrich). Capsules were covered in foil and left to dry overnight. They were then stored in darkness until required. The E_2 capsules were washed with 100% ethanol and bathed in sterile physiological saline overnight prior to implantation. The capsules were placed subcutaneously on the dorsum of the rat through the incision made for ovariectomy. A pair of blunt forceps was then used to create a pocket under the skin and place the capsule subcutaneously. Finally, the skin incision was closed with 3.0 mercerised silk sutures. The animal was then allowed to recover from anaesthesia on a heat pad. Once recovered, the animals were returned to their individual cages.

2.2.2.3 Head screws for tether attachment

Under general anaesthesia, the scalp of the animal was shaved and sterilised and the eyes were protected as described above. The head was placed in a stereotaxic frame (David Kopf Instruments) and a midline incision (approximately 2.5 cm) was made with a scalpel and the skin was retracted. The periosteum was

removed using a scalpel blade and the skull was dried with sterile gauze.

Three screw holes, two in the occipital bone and a third on the right hand side of the frontal bone, were drilled with a dental bur (Ash® Instruments) in a handheld cordless drill (Interfocus). Three stainless steel cheese head screws (10BA × 3/32”; Allscrews) were then screwed into the holes. The slotted screw was secured rostral to the two cheese head screws by dental cement, as described above. The incision was closed around the shaft of the slotted screw using 3.0 mercerised silk. The animal was allowed to recover on a heat pad and returned to their individual cages once recovered.

2.2.2.4 Unilateral cannulation of the left lateral cerebral ventricle

For experiments requiring icv administration of substances, rats were implanted with a chronically indwelling unilateral guide cannula (22 gauge; Plastics One, Roanoke, VA, USA) stereotaxically targeted towards the left lateral cerebral ventricle. The anaesthetised animal was placed in a stereotaxic frame, the scalp was shaved and sterilised, and the skull surface was exposed, as described above. A stereotaxic alignment tool (David Kopf Instruments) was used to align the surface of the skull to ensure that bregma and lambda are in the same horizontal plane (Paxinos and Watson, 1986).

The guide cannula was fitted with an internal cannula (28 gauge; Plastics One), which was attached to a 20 cm length of Tygon tubing (size 0.02; Elkay

Laboratory Products, Basingstoke, UK). The Tygon tubing and internal cannula were filled with artificial cerebrospinal fluid (aCSF) and attached to a 25 µl syringe (Hamilton, Bonaduz, Switzerland) prefilled with sterile water. The guide cannula was secured within a cannula holder tool (David Kopf Instruments) attached to the micro-manipulator, which was then fitted onto the stereotaxic frame. Vertical alignment of the guide cannula was confirmed against a try square positioned on the base of the stereotaxic frame. The tip of the internal cannula was then aimed directly at the bregma to obtain the zero reference anterior-posterior (AP) and medio-lateral (ML) coordinates. The cannula was repositioned 0.6 mm posterior and 1.5 mm left lateral to bregma; coordinates corresponding to the position of the left lateral ventricle according to the Rat Brain Atlas (Paxinos and Watson 1986). Once in position above the left lateral ventricle, a pen mark was made on the skull directly below the internal cannula tip. The manipulator was then moved forward to clear the surface of the skull. A disk bur (FFXC7HP, Ash® Instruments, Dentsply) in a handheld drill was used to create a hole (diameter = 2 mm) in the skull at the above coordinates, and the remaining small piece of bone was carefully removed using watchmaker's forceps (Holborn Surgical & Medical Instruments, Margate, UK). Additionally, two holes in the occipital bone and one hole in the right frontal bone were drilled and three stainless steel cheese head screws (10BA × 3/32") were attached, as described previously. The manipulator arm was then moved back into position with the internal cannula tip aimed at the left lateral ventricle. The zero reference

dorso-ventral (DV) coordinates were obtained by lowering the manipulator until the tip of the internal cannula just made contact with the surface of the dura. The dura was pierced with a hypodermic needle for cannula entry. The cannula was then lowered to 4.5 mm below the zero reference DV coordinates and flushed with 1 µl saline to remove any debris from the tip, before being raised 0.5 mm to give a final cannula position of 4 mm below the surface of the dura. Correct cannula positioning within the lateral ventricle was confirmed by briefly disconnecting the syringe from the distal end of the Tygon tubing and visualising gravitational movement of the meniscus. The guide cannula was then fixed in place using dental cement, as described previously. A slotted screw was affixed, as described above, and dental cement was applied to the three cheese head screws, slotted screw head and guide cannula pedestal, forming a smooth unified assembly. Once the cement had set, the internal cannula was removed from the guide cannula and replaced with a dummy cannula (Plastic One) to maintain patency. The skin was then sutured around the shaft of the slotted screw and guide cannula using 3.0 mercerised silk. The animals were then allowed to recover from anaesthesia on a heat pad until fully conscious.

2.2.2.5 Bilateral cannulation of the brain nuclei

For experiments involving the bilateral administration of substances into the brain nuclei, rats were fitted with a chronically indwelling double guide cannula

(22 gauge; Plastics One) stereotaxically positioned 1 mm above the nuclei, including the mPOA, ARC and MeA. The position information is found in Table 2.1. The procedure was carried out with the aid of a surgical microscope and a fibre-optic cold light source. Anaesthetised rats were prepared for surgery as described above. The guide cannula was fitted with a double internal cannula (28 gauge; Plastics One), which extends 1 mm beyond the tips of the guide cannula to reach the target. The guide/internal cannula assembly was then secured within a cannula holder tool attached to a micro-manipulator, which was then fitted onto the stereotaxic frame. Vertical alignment of the guide cannula was confirmed against a try square positioned on the base of the stereotaxic frame. Coronal alignment was confirmed against the ear bar. The tip of the right internal cannula was positioned directly above the bregma to obtain the zero reference AP and ML coordinates. The cannula was then moved into the correct AP and ML coordinates for implantation (see Table 2.1 for coordinates), such that each guide of the cannula was equidistant from the midline, spanning the dorsal sagittal sinus. Once in position, pen marks were made on the skull directly below the internal cannula tips. The manipulator was then moved forward to clear the surface of the skull. Two overlapping holes or three holes (diameter = 2 mm) in the same coronal plane were drilled in the skull by means of disk bur driven by a handheld drill to expose the entry site for the bilateral cannulae and the dorsal sagittal sinus. The remaining disks of bone were carefully removed using watchmaker's forceps. Three additional holes were drilled and fitted with

stainless steel screws for additional anchorage, as described above. The cannula was then moved back into position, with the right internal cannula positioned directly over the midline of the dorsal sagittal sinus, to obtain final zero reference ML and DV coordinates. The cannulae were then replaced at the specified coordinates such that the internal cannula tips were equidistant from the midline, and able to clear the dorsal sagittal sinus. The dura was lanced directly below the internal cannula tips. The cannula was then carefully lowered, until the tip of the internal cannula reached appropriate distance below the surface of the dura (see Table 2.1 for coordinates). The guide cannula and a slotted screw for later attachment of a metal spring tether were then cemented in place, as described above. Once the cement had set, the guide cannula was released from the micro-manipulator and the skin was sutured around the cannula pedestal and shaft of the slotted screw using 3.0 mercerised silk. The internal cannula was replaced with a dummy cannula and a crystal cap (Plastics One) to maintain patency. Following surgery the animals were allowed to recover from anaesthesia on a heat pad until fully conscious.

Nucleus cannulated	Anterior-posterior (AP)	Medio-lateral (ML)	Dorso-ventral (DV)
ARC	-3.3	±0.5	-10.2
mPOA	-0.3	±0.5	-8.6
MeA	-3.3	±3.2	-8.6

Table 2.1 Coordinates for bilateral cannulation of the ARC, mPOA and MeA. These measurements (mm) were derived from the Rat Brain Atlas by Paxinos and Watson (1986).

2.2.2.6 Bilateral cannulation of the internal jugular veins

After a 10-d post-operative recovery period following ovariectomy, if required, for the tether screw attachment and the cannulation of the left lateral cerebral ventricle, ARC, mPOA or MeA, the animals were implanted with iv catheters for collection of blood samples or systemic administration of injectable substance. The construction of iv catheters was the same as described above expect for the proximal end which was made of Silastic^{TM} medical grade tubing (STHT-C-025-0, Sanitech) suitable for adult rats. The Silastic tubing was cut to length of 28 mm (right side) or 27 mm (left side) measured from the ring of silicone adhesive. The resulting catheters were flushed with sterile saline and a saline-filled 2 ml syringe (Terumo, Egham, UK) was attached to the distal end of the catheter.

Anaesthetised rats were sterilised and ventral surface of the animal's neck was exposed for surgery. Bilateral jugular veins were exposed, incised and inserted by iv catheters. The distal end of the iv catheters were retracted and exteriorised above the head by the aid of the crocodile forceps (Harvard Apparatus). Catheters were later tunnelled through a 30 cm long spring tether (Instec) and filled with 0.2 ml polyvinyl pyrolidone (Sigma-Aldrich). Detailed surgical procedures were described in *2.1.2.3*. The animals were left to recover from surgery for 3 d before experiments commenced.

2.2.3 Experimental procedures

2.2.3.1 Automated serial blood sampling

Each rat was designed to be bled multiple times for the efficiency of animal usage. Rats were bled with administration of vehicle or different dosage of pharmacological agents one time each without repetition. There was a 3-4 d gap between each bleeding procedure and rats were not to be bled more than 4 times.

On the morning of experimentation, the polyvinyl pyrolidone solution was aspirated from one of the two indwelling iv catheters, and the cannula was flushed with physiological saline and connected to one of the channels of a dual-channel swivel (Instec), as described above. Once connected, the animals were left undisturbed for 1 h. Blood sampling commenced at approximately 1000 h, and samples were collected every 5 min for up 6 h.

The custom-made software (Mr D. Layman, King's College London) has the option to collect a smaller amount of blood (25 µl) for adult rats compared with the prepubertal rats for each cycle. The detailed steps in each cycle is the same as the prepubertal one except when the pump flushed the blood through the solenoid into the PP10 tubing it took 10 s instead of 15 s. It caused 25 µl withdraw of blood samples for each cycle instead of 35 µl, making the final volume within each LP-2 tube 175 µl (25 µl blood and 150 µl heparinised saline). A schematic diagram summarising the stages of the automated blood sampling cycle is provided (Figure 2.2).

After the completion of all of the sampling cycles, the iv cannulae were disconnected from the bleeding system and refilled with 0.2 ml polyvinyl pyrolidone, as described above. The system was flushed with sterile water followed by 75% ethanol, each for 10 min and the collected blood samples were frozen at -20°C.

2.2.3.2 Stress paradigms

Restraint stress

Custom-built restraint devices were used for applying restraint stress to rats (Li *et al.* 2004). This was necessary since commercially available restraint devices would not be suitable for mobile animals with the head tether attachment used for the automated blood sampling in the experiment. The restraint devices were

built from strong transparent plastic, in the shape of a cylinder, 18.0 cm in length and 5.8 cm in diameter, with a hinged top to allow placement of the rat inside. The head of the rat protruded through a 3.2 cm diameter collar at one end of the device. At the tail end of the device was a fixture with a 1.5 cm diameter central hole to allow the rat's tail to extend out from the device. The cylinder was equipped with air holes around the whole tube to avoid overheating. The rat was placed into the open restraint tube and the top half of the tube was then closed around the rat's body. The rat was checked to ensure that no skin was caught in the edges of the device and that the head could move freely. The rear plate was pushed into the end of the restraint tube with a hole for the rat's tail. The animal was kept in the device for 1 h.

Immunological stress

After 2-h of blood sampling, lipopolysaccharides (LPS; 15 µg/kg in 0.2 ml saline, Sigma-Aldrich) was administered via the second iv catheter; 0.2 ml sterile saline was flushed along the catheter after the LPS to ensure complete delivery of LPS. Control animals were administered with 0.2 plus 0.2 ml sterile saline.

2.2.3.3 Central microinjections

For the purpose of administration of drugs into the brain structures, including the lateral cerebral ventricular and the nucleus (nuclei), rats were attached by the guide cannulae to remote injection systems which were preloaded with drugs or vehicle control, thus allowing remote infusion without disturbing the rat during the experiment. The rat was equipped with a remote injection system at the same time as the attachment to the automated blood sampling system.

Unless otherwise stated, all compounds infused into the brain were dissolved in aCSF, which was made up as follows:

100 ml deionised water	0.214 g $NaHCO_3$
0.724 g NaCl	0.18 g D-Glucose
0.246 g KCl	0.0368 g $CaCl_2$
0.0163 g KH_2PO_4	0.025 g $MgSO_4$

Each salt was added to the deionised water until the previous one had fully dissolved. The aCSF solution was then filtered through a sterile filter (Millipore, Watford, UK), aliquoted and stored at -20ºC.

Unilateral infusion into the lateral cerebral ventricle

For central administration of substance into the brain, animals were attached through the icv guide cannula to a remote injection system. A unilateral icv

internal cannula (Plastic One) was attached to a 30 cm length of Tygon tubing (ID: 0.51 mm, OD: 1.53 mm; Elkay). The distal end of the Tygon tubing was connected to the dual-channel swivel on the second channel not used for the automated blood sampling system. A 5 cm length 0.02 Tygon tubing was then attached to the distal side of the dual-channel swivel. The whole system was filled with aCSF using a 1 ml syringe. Approximately 2 µl air was placed in the extensive Tygon tubing to determine whether fluid was in fact passing down the cannula. The 1 ml syringe was then replaced by a 25 µl Hamilton syringe (Waters International, UK) primed with sterile water. Air (1 µl) was drawn into the internal cannula to separate the drug and aCSF, followed by 4 µl of the solution to be administered and another 1 µl of air. The position of the 2 µl air bubble within the extension tubing was then marked to indicate the pre-injection reference point. The dummy cannula was gently removed from the guide cannula and replaced with the internal cannula, which was held in place using a plastic applicator cap with a hole to allow the 30 cm Tygon tubing to pass through it. The Tygon tubing was then taped to the tether spring out of the animal's reach. Injections were performed manually at a constant speed over 5 min, and the displacement of the 2 µl air bubble was used to track the progress of the injections. At the end of the experiment, the internal cannula was removed and replaced with a dummy cannula and crystal cap.

Bilateral infusion into the nuclei

For bilateral administration of drugs into the brain nuclei, the rat was attached by the double guide cannulae to a remote injection system. The system comprised a bilateral internal cannula (Plastics One), each channel of which was attached to an 80 cm length of extension tubing (Portex PP50 tubing); the distal ends of PP50 tubing were attached to a 5 cm length of Tygon tubing transiently expanded by immersion in xylene. The system was filled with aCSF and a 5 µl syringe (Hamilton) primed with sterile water was connected to each distal end of the assembly via the Tygon tubing. 1 µl air bubbles were loaded into the extension tubing to monitor progress of administration in response to fine movement of the syringe plungers. Air (0.2 µl) was drawn into the internal cannula to separate the drug from the upstream aCSF, followed by 0.4 µl of the solution to be administered and another 0.2 µl air. The Hamilton syringes were taped together and then securely taped to the front of the cage at approximately the same level as the rat to prevent premature leakage of the solution into the brain due to gravity. The dummy cap and cannula were gently removed from the guide cannula and the internal cannula was then fully inserted into the guide cannula. The internal cannula was held in place using a plastic applicator cap with a hole to allow passage of PP50 tubing. The PP50 tubing was then taped to the tether spring out of the animal's reach. Injections were performed manually at a constant speed over 5 min, and the displacement of the 1 µl air bubble was used to track the progress of the injections. At the end of the experiment, the

internal cannula was removed and replaced with a dummy cannula and crystal cap.

2.3 Radioimmunoassay for determination of LH levels

A double-antibody radioimmunoassay supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, Bethesda, MD, USA) was used to determine LH concentrations in the whole blood samples. The sensitivity of the assay was 0.093 ng/ml.

2.3.1 Reagents used in the radioimmunoassay

General Diluent:	1400 ml	Deionised water
	8.09 g	Na_2HPO_4 (anhydrous) (Sigma-Aldrich)
	2.03 g	$NaH_2PO_4 \cdot 2H_2O$ (Sigma-Aldrich)
	0.14 g	Thimerosal (Sigma-Aldrich)
	7.00 g	Bovine Serum Albumin (BSA; Sigma-Aldrich)

The above components were dissolved in 1000 ml water in order, with each component only added once the previous had fully dissolved. A further 400 ml of water was then added to bring the total volume to 1400 ml and give a 0.05 M phosphate buffer (pH 7.4) with 0.5% BSA. This buffer was then used for

iodination, preparation of standards, primary antibody diluent, label and precipitating antibody buffer.

Primary antibody diluent:	45 ml	General diluent
	0.84 g	EDTA
	149.5 µl	Normal Rabbit Serum

Primary antibody:

The lyophilised antibody NIDDK-anti-ratLH-S-11 was used as a primary antibody for the LH radioimmunoassay. The lyophilised protein was reconstituted in 1 ml of deionised water to give a buffered solution with an antibody dilution of 1:40. The antibody was then split into 25 µl aliquots and stored at –20˚C. For use in the assay aliquots were thawed and diluted with 1 aliquot per 45 ml of primary antibody diluent.

Precipitating antibody:

Anti-rabbit gamma globulin raised in the donkey was used (IDS, Boldon, UK). For use in the assay this was diluted 1:100 in general diluent.

Iodine^{125}-labelled LH:

Rat LH was labelled with radioactive iodine-125 (^{125}I) by means of an iodination procedure similar to that described previously (Markwell and Fox 1978) (See sections 2.3.2 and 2.3.3).

Rat LH standards:

The referenced preparation used for the standard curve was NIDDK-rLH-RP-3 (5 μg ampoule) supplied in lyophilised form. The protein was dissolved in 1 ml of deionised water. The protein was then stored in 40 μl aliquots (containing 200 ng LH) at -20°C until required. The highest concentration standard (16 ng/ml) was produced by diluting one aliquot of rat LH in 12.46 ml general diluent. The resulting 16 ng/ml standard was then split into 400 μl aliquots and stored at -20°C until required. On the first d of an assay, one aliquot of the 16 ng/ml standard was removed from the freezer. 200 μl of the standard was then used to in a 1/2 dilution series to produce the remaining standards containing 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.01562 and 0 ng/ml rat LH. Three LP2 tubes were then labelled S1 to S12, for each of the 12 standard curve concentrations. 50 μl each of the 12 dilutions of rat LH standard was dispensed in triplicate into pre-labelled LP-2 tubes. A further 50 μl of saline was then added to all standard curve tubes before being used immediately in the assay.

Positive controls:

In order to calculate the coefficient of variation across multiple repetitions of the LH RIA, samples of pooled plasma, obtained by centrifugation of whole blood from OVX rats, were aliquoted into 25 µl,12.5 µl, 6.25 µl volumes and made up to 100 µl with general diluent. These control samples were run in triplicate in each LH RIA, with one set of plasma pools added to the assay approximately every 500 tubes to account for potential drift.

Washing solution:	1000 ml	Deionised water
	40 g	Polyethylene glycol (PEG; Sigma-Aldrich)
	9 g	NaCl (Sigma-Aldrich)
	2 ml	Triton® X-100 (Sigma-Aldrich)

The above components were dissolved in 800 ml water in the specified order, with each component only added once the previous had fully dissolved. A further 198 ml water was then added to bring the total volume to 1000 ml. The resulting solution is 0.9% saline with 4% PEG and 0.2% Triton® X-100 detergent.

2.3.2 Reagents used in the iodination of rat LH

Purified rat LH:

Iodination grade protein NIDDK-rLH-1-9 was supplied by the NIDDK in lyophilised form. It was reconstituted with 200 µl of 0.5 M phosphate buffer. Aliquots of 10 µl (containing 5 µg rat LH) were then stored at -80°C until required for iodination.

Idoine-125:

37 MBq iodine-125 (^{125}I) was supplied by Perkin-Elmer (Cambridge, UK) in the form of 10 µl $Na^{125}I$.

Phosphate Buffer (PB) 0.5 M (pH7.4):	140 ml	Deionised water
	7.1 g	Na_2HPO_4 (anhydrous)
	3.12 g	$Na_2HPO_4 \cdot 2H_2O$
Phosphate Buffer (PB) 0.05 M (pH7.4):	1400 ml	Deionised water
	7.1 g	Na_2HPO_4 (anhydrous)
	3.12 g	$Na_2HPO_4 \cdot 2H_2O$

Iodination vials:

The catalytic matrix formed within the iodination vials was used as a reducing

substrate for the ^{125}I. 1 mg Iodo-Gen® (1,3,4,6-Tetrachloro-3a, 6a-diphenylglyvouril: Sigma-Aldrich) was dissolved in 10 ml chloroform (Sigma-Aldrich). This solution (50 µl) was then placed into the bottom of small glass test tubes (Thermo Life Sciences). The tubes were left at room temperature until the chloroform had evaporated away leaving the matrix of iodogen at the bottom of the tube. The iodogen coated vials were then sealed with a small rubber stopper and stored at -20°C until required.

2.3.3 Procedure for iodination of rat LH

This procedure was carried out behind a Perspex shield positioned within a ventilated cupboard certificated for work involving radioactivity. 5 g Sephadex G-75 (Sigma-Aldrich) was soaked overnight in 150 ml deionised water at 4°C. On the morning of the iodination, the Sephadex was allowed to warm to room temperature. The top of a 10 ml polystyrene serological pipette (BD Falcon, Oxford, UK) was sawn off. The tapered end of the pipette was then plugged with approximately 2 cm of glass wool (Surechem Products, Needham Market UK). One aliquot (5/10 µl) of rat LH was diluted with 20 µl of 0.5 M PB and mixed. The entire 30 µl was then added to the bottom of the iodination vial. 5 µl ^{125}I (equivalent to 17.5 MBq) was then immediately added to the iodination vial. The mixture was then left for 30-40 min to allow the reduction of molecular $Na^{125}I$ to $^{125}I^-$, and the subsequent binding of the ionic iodine-125 to tyrosine residues of

the rat LH protein. During the 30-40 min reaction period the elution column was made.

The prepared 10 ml plastic pipette was fixed vertically in a clamp stand with the tapered end plugged with glass wool at the bottom. Soaked Sephadex G-75 was slowly added to the pipette with deionised water in order to prevent the Sephadex from drying. Soaked Sephadex was added to the column and allowed to settle, while the water fraction was allowed to flow out of the column. More water was constantly added to the column to prevent the Sephadex from drying. Once the Sephadex had settled and formed a distinct meniscus, at least 10 ml of general diluent was run through the column, still ensuring the Sephadex was not exposed to air. After 30-40 min the reaction between ^{125}I and rat LH was quenched by the addition of 150 µl 0.05 M PB. General diluent was allowed to run through the elution column until the meniscus of the buffer had reached the top of the Sephadex layer. At this point, whilst being careful not to allow the Sephadex to come into contact with air, the reaction product was transferred from the iodination vial to the elution column. The position of the radioactivity within the column was judged using a hand-held Geiger counter (Mini 900; Perspective Instruments, Shaw, UK). Once the radioactivity reached approximately halfway through the column, a series of 40 500 µl fractions of eluent were collected into pre-labelled 1.5 ml tubes (Eppendorf, Stevenage, UK). During this collection period the column was constantly topped up with general diluent. 10 µl from each collected fraction was transferred into clean pre-labelled

LP-3 tubes, which were counted for 1 min using an automatic gamma counter (WIZARD^2; Perkin-Elmer) to determine the fractions that contained iodinated rat LH (^{125}I-rLH).

Figure 2.3 illustrates a typical iodination profile. The fractions represented by the first peak contain ^{125}I-rLH. Any tubes from the first peak with counts per minute over approximately 1,000,000/10 µl were mixed and diluted with general diluent to give approximately 85,000 cpm/µl. Aliquots of 100 µl were then placed into 0.5 ml Eppendorf tubes and stored at -20°C until required.

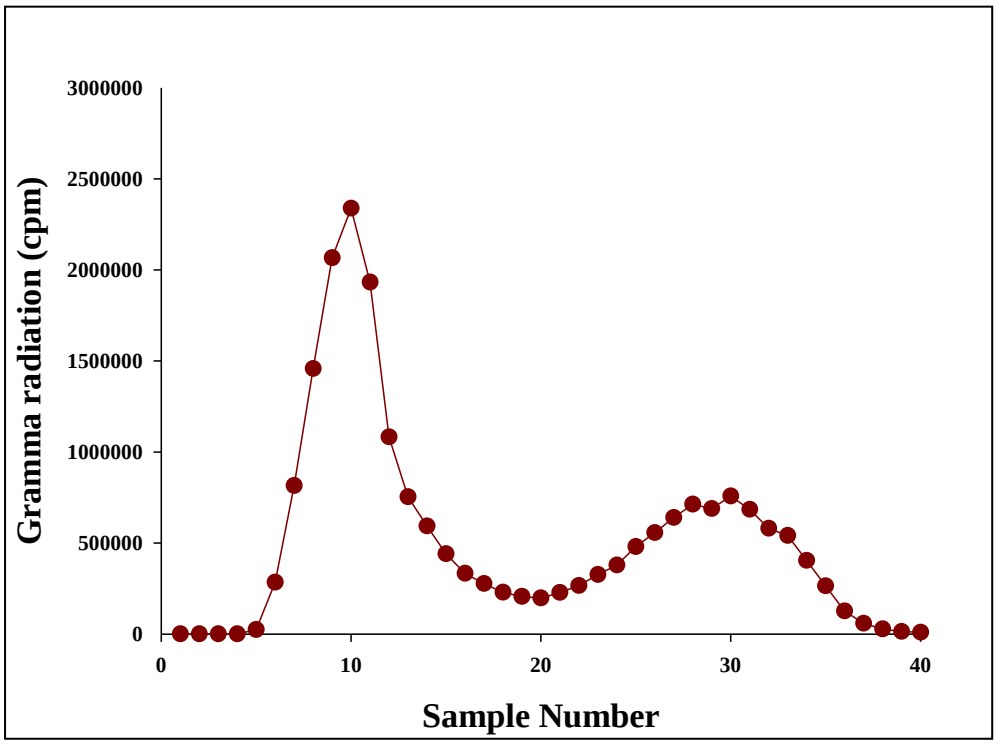

Fig. 2.3 Representative examples of an elution profile from a Sephadex G-75 column following the iodination reaction of 5 µg rat LH with 18.5 MBq ^{125}I.

2.3.4 Procedure for the radioimmunoassay

The assay was carried out over 4 consecutive d. Reagents were added at room temperature, but otherwise samples were stored at 4°C. The sequence of samples assayed was as follows:

Tubes 1-3: Total counts tubes

Tubes 4-6: Non-specific binding tubes

Tubes 7-42: Standard curve; 12 standard concentrations, run in triplicate, starting with the standard of lowest concentration

Tubes 43-51: Plasma pools

Tubes 52-end: Blood samples of unknown LH concentration. Additional sets of plasma pools were added approximately every 500 samples.

D 1:

1. 50 µl primary antibody diluent was added to each of the three non-specific binding tubes in the standard curve.
2. All other tubes, with the exception of the total counts tubes, received 50 µl of primary antibody diluted in primary antibody, as described above.
3. All tubes were vortexed and stored overnight at 4°C.

D 2:

4. An aliquot of ^{125}I-rLH was diluted with general diluent to 150-200 cpm/µl. 50 µl of the resulting solution was dispensed into the total counts tubes, which were then counted using an automatic gamma counter to confirm adequate radioactivity levels (7,500-10,000 cpm/tube).

5. Each tube received 50 µl ^{125}I-rLH diluted to 150-200 cpm/µl.

6. All tubes were vortexed and stored overnight at 4°C.

D 3:

7. All tubes, with the exception of total counts tubes, received 50 µl precipitating antibody diluted with general diluent, as described above.

8. All tubes were vortexed and stored overnight at 4°C.

D 4:

9. All tubes were centrifuged at 3,500 rpm for 60 min at 4°C.

10. Excluding the three total counts tubes, the supernatant in each tube was aspirated away using a water suction aspirator, without disturbing the pellet, and discarded.

11. Excluding the three total counts tubes, each tube received 200 µl washing solution and were again centrifuged at 3,500 rpm for 60 min at 4°C.

12. Excluding the three total counts tubes, the supernatant in each tube was again aspirated using a water suction aspirator, without disturbing the pellet, and discarded.

13. The radioactivity in the remaining pellets at the bottom of each tube was then counted for 1 min per tube using an automatic gamma counter. Tubes were counted in the order previously specified. The standard curve was automatically plotted by the computer software, and the unknown concentrations of LH were calculated from this curve.

The detection limit of the LH RIA was 0.093 ng/ml and the three plasma pool concentrations were 8.41 ± 0.07, 4.64 ± 0.18 and 2.05 ± 0.20 ng/ml (mean ± SEM). The intra-assay variation was 5.8%, and the inter-assay variation was 7.2%.

2.3.5 LH pulse detection

The algorithm ULTRA was used to aid the detection of LH pulses (Van Cauter 1988). Two intra-assay coefficients of variation of the assay were used as the reference threshold for the pulse detection. Briefly, ULTRA functions by comparing each data point to the two points before and after it, and determining if a significant rise and fall has occurred in the profile; this is consistent with a pulse. To allow for the fact that a pulse may occur over several data points, the

data is filtered so that only absolute troughs and peaks are present; any intermediate points are disregarded. If the difference between any trough and peak is greater than two coefficients of variation, the peak is considered a pulse.

CHAPTER THREE: NK3R ANTAGONISM DECREASES LH PULSE FREQUENCY AND AMPLITUDE AND DELAYS PUBERTY ONSET IN FEMALE RATS

3.1 Introduction

The reproductive system is regulated by an intricate network of central and peripheral factors; however, the precise mechanisms triggering critical reproductive events, such as puberty onset, remain mostly unknown. Recently, a plethora of studies have revealed Kiss1 and NKB as gatekeepers of puberty and essential components of normal reproductive function. Humans with inactivating mutations in *TAC3* or *TACR3*, the genes encoding NKB or its receptor NK3R, respectively, present with severe congenital gonadotrophin deficiency and pubertal failure (Topaloglu *et al.* 2009). Individuals with this rare recessive mutation disorder show low levels of sex steroids, LH and underdeveloped gonads (Young *et al.* 2010). Analysis of gonadotrophin concentrations in patients with *TAC3* or *TACR3* mutations showed very weak LH secretion with reduced frequency and amplitude of LH pulses, whereas the FSH concentrations is preserved (Francou *et al.* 2011). In those patients, normal LH pulses were induced by pulsatile administration of exogenous GnRH (Young *et al.* 2010, Francou *et al.* 2011).

Thus, the interest in deciphering the role of NKB-NK3R signalling in the central control of the reproductive system arose. However, initial studies involving the

action of NKB upon gonadotrophin release has been controversial, with some showing the suppressive effect on LH secretion (Sandoval-Guzman and Rance 2004, Navarro *et al.* 2009a, Wakabayashi *et al.* 2010, Kinsey-Jones *et al.* 2012), with others showing the opposite effect with NKB or its agonist stimulating LH release across species (Billings *et al.* 2010, Ramaswamy *et al.* 2010, Navarro *et al.* 2011a, Navarro *et al.* 2011b). Although speculative, this discrepancy might be due to the differences in the species, sex and gonadal status. Nonetheless, in prepubertal stage, acute administration of senktide induced LH pulses in monkeys (Ramaswamy *et al.* 2011), rats (Grachev *et al.* 2012b) and sheep (Nestor *et al.* 2012), suggesting NKB-NK3R signalling as a stimulative component before puberty onset, which is in accordance with human genetic studies.

In rodents, *Tac2* and *Tacr3* (encoding NKB and NK3R) mRNA is widely expressed throughout the brain including amygdala, bed nucleus of the stria terminalis and ARC. In adult rodents, NKB-NK3R signalling in the ARC has been implicated in gonadal steroids negative feedback control by virtue of up-regulated *Tac2* mRNA in the presence of gonadal steroids and down-regulated *Tac2* mRNA in gonadal steroids deprivation situations, such as post-gonadectomy (Danzer *et al.* 1999, Dellovade and Merchenthaler 2004, Navarro *et al.* 2009a, Navarro *et al.* 2011a). Similarly, in the prepubertal stage, NKB-NK3R signalling is also involved in gonadal steroids negative feedback control, at least in female mice (Kauffman *et al.* 2009). Despite of that, the major

regulation pathway that drives the stimulation of NKB expression prior to puberty onset seems to dominate over the rising levels of gonadal steroids that occur during puberty onset. It is supported by the fact that the level of expression of *Tac2* in ARC increases gradually across sexual maturation and peaks at puberty onset, despite the increased circulating E_2 levels during that period (Gill *et al.* 2012, Li *et al.* 2012, Navarro *et al.* 2012). Furthermore, to determine whether the NKB-NK3R signalling has a stimulatory role on puberty onset, the effects of pharmacologic blockade with an NK3R antagonist (SB222200) on puberty onset were investigated (Gill *et al.* 2012, Navarro *et al.* 2012). Central administration of SB222200 tends to modestly delay puberty onset in rats (Navarro *et al.* 2012), whereas peripheral administration of SB222200 has no significant impact on puberty onset in mice (Gill *et al.* 2012). This discrepancy could be due to differences in species and methodologies between studies. Surprisingly, the latter study is consistent with a finding of genetic knockout mice lacking the *Tacr3* genes that displayed normal timing of puberty onset (although with abnormal oestrous cycles and other reproductive phenotypes) (Yang *et al.* 2012). Thus, the role of NKB-NK3R signalling in the developmental reproductive system remains to be further investigated in animal studies.

Furthermore, the precise mechanisms by which NKB-NK3R signalling exerts its effects on the central control of the reproductive system remain to be established. A necessity for the initiation of puberty is the acceleration of the GnRH pulse generator, resulting in the concomitant increase in LH pulse frequency (Sisk and

Foster 2004, Plant 2008, Mayer *et al.* 2010, Li *et al.* 2012). The exact neuronal component of the GnRH pulse generator and the mechanisms underlying its acceleration are still unknown. The discovery of Kiss1 and its receptor, Kiss1r, has yielded substantial evidence that Kiss1-Kiss1r signalling plays a critical role in initiating puberty and maintaining normal reproductive function (de Roux *et al.* 2003, Seminara *et al.* 2003, Kirilov *et al.* 2013). Across many species, Kiss1 neurones in the ARC co-express NKB and Dyn and are named the KNDy neurones (Lehman *et al.* 2010). It is proposed that NKB is stimulatory to KNDy neurones, whereas Dyn is inhibitory (Navarro *et al.* 2009a, Wakabayashi *et al.* 2010). It has been shown that KNDy neurones in the ARC express NK3R and project to one another forming a network where they communicate via this receptor (Krajewski *et al.* 2010). This network may represent part of the neuronal component of the GnRH pulse generator (Rance *et al.* 2010, Okamura *et al.* 2013). Thus, it is reasonable to speculate that ARC NKB-NK3R signalling is involved in the acceleration of pulsatile GnRH release triggering puberty onset.

Reproduction is an energy-demanding function which is subjected to regulation by metabolic cues. Previous studies have implicated Kiss1 in this process, with reduced hypothalamic *Kiss1* gene expression and LH secretion in prepubertal rats in acute fasting (Castellano *et al.* 2005). Given the fact that NKB-NK3R signalling is highly integrated with Kiss1 signalling in the context of KNDy neurones, it is reasonable to assume that NKB is also involved in metabolic

stress-induced reproductive dysfunction. In this sense, negative energy balance decreases NKB and NK3R expression in the hypothalamus and delays puberty onset in female rats, which can be rescued by administration of NKB (Navarro *et al.* 2012). Furthermore, in this study, the stimulative effect of NKB on LH secretion in prepubertal rats is not only preserved but augmented, indicating a sensitized effect of NKB in negative energy balance (Navarro *et al.* 2012). However, less attention has been paid to neural mechanisms in over-nutrition states, which induce obesity and advance puberty onset. Childhood obesity, which has escalated in recent decades (Ogden *et al.* 2002, Wang and Lobstein 2006, Ogden *et al.* 2012), is not only related to cardiovascular and metabolic risk and to adulthood obesity, but can also lead to earlier onset of puberty, which in girls is a risk factor for anti-social behaviour, anxiety and other mental health disorders, in additions to gynaecological disorders and cancers consequent to earlier sexual activity and first pregnancy (Apter and Vihko 1983, Michaud *et al.* 2006, De Leonibus *et al.* 2012). In animal models, for example, rats fed a high fat diet, demonstrate increased body weight gain and earlier sexual maturation (Li *et al.* 2012, Fungfuang *et al.* 2013). This precocious puberty may be related to the premature activation of the GnRH pulse generator and hence increased LH pulse frequency (Li *et al.* 2012). The up-regulation of *Tac2* expression in the ARC of rats fed a high fat diet suggests that NKB-NK3R signalling may also be involved in obesity-induced puberty advancement and premature activity of GnRH pulse generator (Li *et al.* 2012).

3.2 Aims

To test the hypothesis that endogenous NKB-NK3R signalling regulates the timing of puberty onset and the dynamic pulsatile release of LH during the prepubertal stage in female rats.

a) To investigate the role of endogenous NKB-NK3R signalling in the timing of puberty onset in female rats, including the precocious puberty onset induced by over-nutrition status.

b) To investigate the mechanisms of endogenous NKB-NK3R signalling involved in the process of puberty onset by determining changes in LH pulse frequency in prepubertal female rats administered NK3R antagonist, in both normal and over-nutrition status.

3.3 Materials and methods

3.3.1 Animals and surgical procedures

Adult Sprague-Dawley rats were allowed to mate to produce litters (see section 2.1.1). Litters were either assigned to a standard chow diet or a high fat diet after weaned at pnd 21 (see section 2.1.1).

All surgeries were carried out under a combination of ketamine and xylazine (see section 2.1.2). On pnd 24 (Fig. 3.1), standard chow diet fed female rats were chronically implanted with icv cannulae. Animals were fitted with a 28 gauge

cannula (Plastics One, Roanoke, VA, USA) directed towards the left lateral cerebral ventricular: the coordinates for implantation were 1.2 mm lateral, 1 mm posterior to bregma, and 4.5 mm below the surface of the dura according to the Rat Brain Atlas of Paxinos and Watson (Paxinos G 1986). An osmotic mini-pump (Model 2002, Alza Corp, Mountain View, CA, USA) was attached to the cannula with silicone tubing, and implanted subcutaneously in the interscapular space. The NK3R antagonist SB222200 (dissolved in aCSF/15% DMSO/20% cyclodextrin) (Sarau *et al.* 2000) or aCSF (contains 15% DMSO and 20% cyclodextrin) was delivered via the osmotic mini-pump at 0.5 µl/h for a 14-d period. The dose of SB222200 used was 2 nmol/µl (24 nmol/d). On pnd 31 (Fig. 3.1), female rats were fitted with an indwelling cardiac catheter via the right jugular vein (see section 2.1.2.3). The free end of the catheter was exteriorised at the back of the head. A small metal screw was fixed by two small screws anchored to the occipital bones with dental cement (see section 2.1.2.3). A 30 cm long lightweight spring tether (Instec Laboratories Inc., Boulder, CO) was connected to the metal screw to protect exteriorised cardiac catheters. The distal end of the tether was attached to a fluid swivel (Instec Laboratories), which allowed the rat freedom to move around the enclosure. Rats were housed separately after surgery and allowed 3-d of recovery before experimentation (Fig. 3.1).

Female rats fed the high fat diet were also fitted with icv cannulae on pnd 23 and received SB222200 or aCSF (as above) via an osmotic mini-pump for 14 d. On

pnd 29, rats were fitted with an indwelling cardiac catheter via the right jugular vein and experiments started 2 d later (Fig. 3.1).

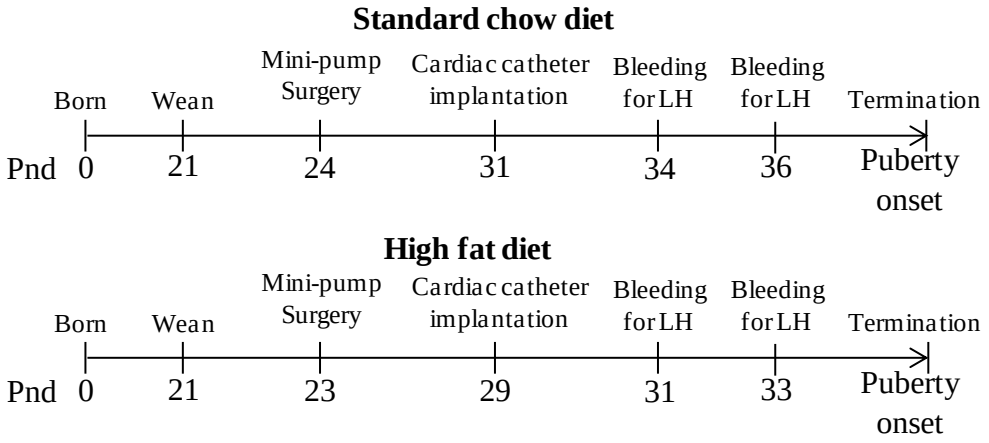

Fig. 3.1 Timeline diagram of experimentation with prepubertal rats fed with either standard chow diet or high fat diet.

3.3.2 Experiment 1: Effects of NK3R antagonism on the timing of puberty onset in female rats fed a standard chow diet or a high fat diet

Litters were weaned on pnd 21 and housed in groups of 3 to 4 per cage until they underwent surgical procedures, after which they were individually caged. Rats provided with the standard chow diet were implanted with icv cannulae connected to osmotic mini-pumps filled with either aCSF or SB222200 (n = 9-12 per group) on pnd 24, as described above. Similarly, rats provided with the high fat diet were implanted with icv cannulae connected to osmotic mini-pumps filled with either aCSF or SB222200 (n = 10-12) on pnd 23. Rats were weighed

every 3 d after pnd 25. They were also monitored daily for vaginal opening and the first vaginal oestrus (markers of puberty onset) after pnd 28. Vaginal opening were assessed approximately at 0900 hour (h) every d. Once vaginal opening occurred, vaginal smears were taken daily at 0900 h to detect the stage of the oestrous cycle (Goldman *et al.* 2007) until the first oestrous cycle was detected.

3.3.3 Experiment 2: Effects of NK3R antagonism on LH pulse parameter in female rats fed a standard chow diet

Blood collecting procedures were carried out on pnd 34 and 36 in rats fed with a standard chow diet (n = 6-7 per group), in an attempt to capture a changing LH pulse frequency leading to puberty (Li *et al.* 2012). On the morning of experimentation, rats were attached via the cardiac catheter to a computer-controlled automated blood sampling system, which allows for the intermittent withdrawal of small blood samples (35 µl) without disturbing the animals. Once connected, animals were left undisturbed for 2 h before sampling commenced. Blood sampling commenced between 1100 h and 1200 h. Samples were collected every 5 min for 4 h for LH measurement. After removal of each 35 µl blood sample, an equal volume of heparinized saline (50 U/ml normal saline; CP Pharmaceuticals, Wrexham, UK) was automatically infused into the animal to maintain patency of the catheter and blood volume. Blood samples were frozen at -20°C for later assay to determine LH concentrations. After each

bleeding procedure, rats were replaced with 1.5 ml whole blood, iv over 15 min. A further 1 ml of whole blood was administered iv over 10 min the following d to preserve the hematocrit and maintain blood volume.

3.3.4 Experiment 3: Effects of NK3R antagonism on LH pulse parameter in female rats fed a high fat diet

In rats fed a high fat diet, blood collecting procedures were carried out on pnd 31 and 33 (n = 7-9 per group), in keeping with the advanced timing of puberty onset in the over-nutrition status (Li *et al.* 2012). The rest of the experimental procedures were identical to the ones with standard chow diet described above. Rats were continuously bled for 4 h without disturbance. Small amounts of blood (35 µl each) were intermittently collected for later LH assays to determine the LH pulses.

3.3.5 Statistical analysis

Detection of LH pulses was established by use of the algorithm ULTRA. Two intra-assay coefficients of variation of the assay were used as the reference threshold for pulse detection. The effect of SB222200 on parameters of LH secretion was calculated by comparing the mean number of LH pulses or mean amplitude of LH pulses within the 4-h experimental period between the two

groups. Comparisons between SB222200 and control groups on body weight, time of vaginal opening, and first oestrus were made by subjecting data to one-way ANOVA and the Dunnett's test. Comparisons between SB222200 and control groups on LH pulse frequency and amplitude, as well as the comparisons between the body weight of standard chow diet and high fat diet on pnd 31 were made using a two-way ANOVA and the Holm-Sidak post-hoc test. All data are shown as mean ± S.E.M. $P < 0.05$ was considered statistically significant.

3.4 Results

3.4.1 Experiment 1: Effects of NK3R antagonism on the timing of puberty onset in female rats fed a standard chow diet or a high fat diet

To investigate the role of NKB-NK3R signalling in the control of pubertal timing, we examined the effect of centrally administered NK3R antagonist via osmotic mini-pumps on the timing of vaginal opening. In female rats fed a standard chow diet, central (icv) administration of the NK3R antagonist (SB222200, 24 nmol/d) significantly delayed the d of vaginal opening compared with that in the control group by approximately 4 d (aCSF *vs.* SB222200: 37.4 ± 0.5 *vs.* 41.2 ± 0.6 d; $P < 0.05$) (Fig. 3.2, B). The d of first oestrus was also significantly delayed in the treatment group (aCSF *vs.* SB222200**:** 37.6 ± 0.5 *vs.* 41.9 ± 0.9 d; $P < 0.05$). In rats fed a standard chow diet, chronic infusion of

SB222200 had no effect on body weight gain compared to controls (Fig. 3.2, A). In rats fed a high fat diet, icv administration of SB222200 (24 nmol/d) significantly delayed the d of vaginal opening compared to controls by approximately 2 d (aCSF *vs.* SB222200: 33.4 ± 0.4 *vs.* 35.1 ± 0.6 d; $P < 0.05$) (Fig. 3.2, D). The d of first oestrus was also significantly delayed in the treatment group (aCSF *vs.* SB222200: 33.7 ± 0.6 *vs.* 35.9 ± 0.5 d; $P < 0.05$). In rats fed a high fat diet, chronic infusion of SB222200 had no effect on body weight gain compared with controls (Fig. 3.2, C). Furthermore, on pnd 31, the body weight of rats fed with high fat diet was significantly higher than that of rats fed with standard chow diet (aCSF + standard chow diet *vs.* aCSF + high fat diet: 91.2 ± 2.6 *vs.* 113.0 ± 3.5; $P < 0.05$; SB222200 + standard chow diet *vs.* SB222200 + high fat diet: 93.1 ± 3.1 *vs.* 117.0 ± 3.5; $P < 0.05$).

3.4.2 Experiment 2: Effects of NK3R antagonism on LH pulse parameter in female rats fed a standard chow diet

To further determine the mechanisms of NKB-NK3R signalling involved in the timing of puberty onset, serial blood samples were collected from prepubertal female rats fed a standard chow diet to measure LH pulses. The number of LH pulses in the 4-h sampling period increased between pnd 34 and 36 in the control group (Figs. 3.3, A, C and E) and was significantly reduced in rats administered with SB222200 (Figs. 3.3, B, D and E) (number of LH pulses on pnd 36, aCSF

vs. SB222200: 2.5 ± 0.2 *vs.* 1.6 ± 0.2; $P < 0.05$). LH pulse amplitude was significantly reduced in SB222200-treated rats compared with controls on both pnd 34 and 36 (amplitude of LH pulses on pnd 34, aCSF *vs.* SB222200: 3.2 ± 0.4 *vs.* 1.8 ± 0.4; $P < 0.05$; amplitude of LH pulses on pnd 36, aCSF *vs.* SB222200: 2.8 ± 0.2 *vs.* 1.8 ± 0.3; $P < 0.05$) (Fig. 3.3, F).

3.4.3 Experiment 3: Effects of NK3R antagonism on LH pulse parameter in female rats fed a high fat diet

Serial blood samples were also collected in prepubertal female rats fed a high fat diet. The number of LH pulses in the 4-h sampling period increased between pnd 31 and 33 in the control group (Figs. 3.4, A, C and E) and was significantly reduced in rats administered with SB222200 compared with controls on pnd 33 (number of LH pulses on pnd 33, aCSF *vs.* SB222200: 3.6 ± 0.6 *vs.* 2.1 ± 0.4; $P < 0.05$) (Figs. 3.4, C-E). LH pulse amplitude was significantly reduced in SB222200-treated rats compared with the control on pnd 31 (amplitude of LH pulses on pnd 31, aCSF *vs.* SB222200: 3.0 ± 0.4 *vs.* 1.9 ± 0.2; $P < 0.05$) (Figs. 3.4, A, B, and F).

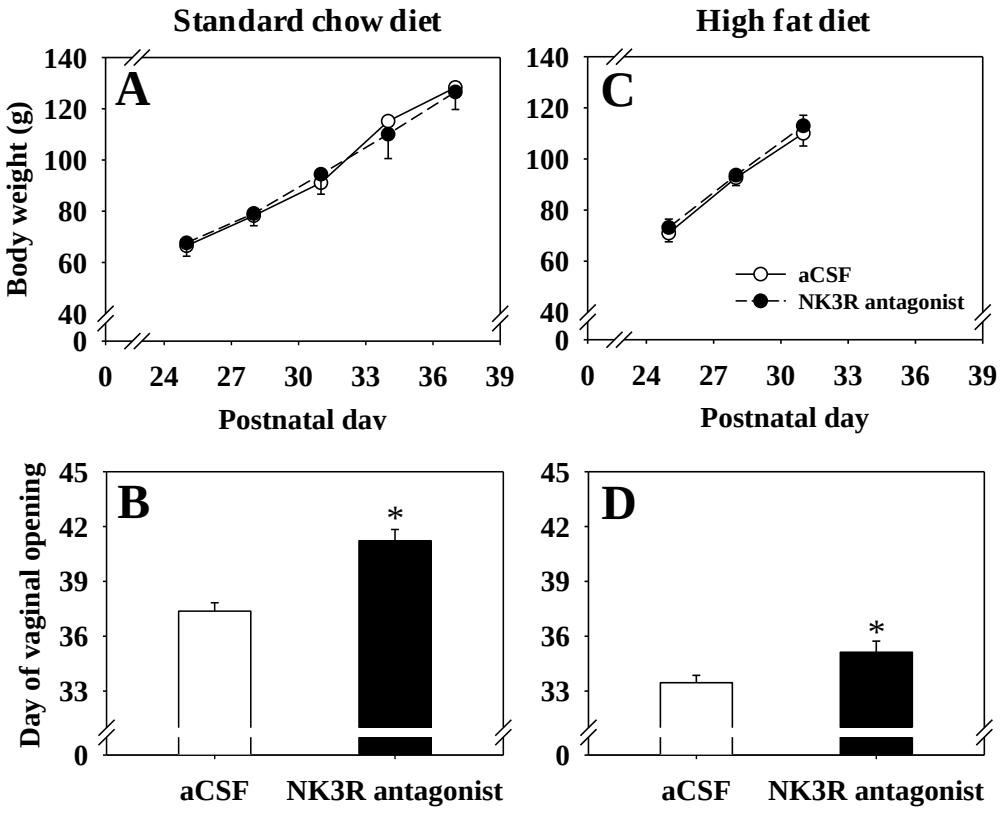

Fig. 3.2 Effects of a NK3R antagonist (SB222200) on body weight and d of vaginal opening in female rats fed with a standard chow or a high fat diet (45% kcal fat intake) after weaned on pnd 21. In rats fed a standard chow diet, the central administration of SB222200 (24 nmol/d, 12 µl/d, icv) had no affect on body weight gain (A) but delayed puberty onset (B) compared to controls (aCSF, 12 µl/d). In rats fed a high fat diet, the central administration of SB222200 (24 nmol/d, 12 µl/d, icv) did not affect the body weight gain (C) but delayed puberty onset (D) compared to controls (aCSF, 12 µl/d). *P < 0.05 *vs.* aCSF group (n = 9-12 per group).

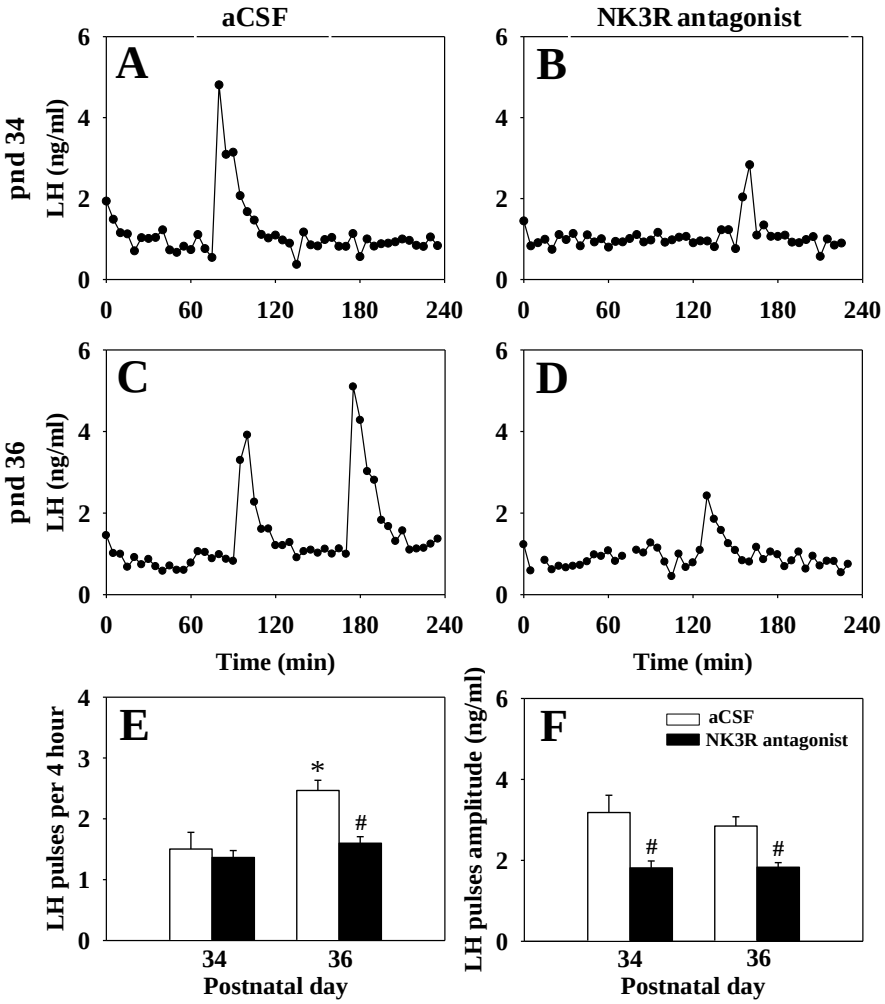

Fig. 3.3 Effects of a NK3R antagonist (SB222200) on LH pulses in prepubertal female rats fed a standard chow diet. (A, C) Representative examples of LH profiles on pnd 34 and 36, respectively, in animals administered aCSF (12 µl/d, icv) as vehicle. (B, D) Representative examples of LH profiles on pnd 34 and 36 in animals administered SB222200 (24 nmol/d). (E) Summary showing that the administration of SB222200 significantly reduced the number of LH pulses in the 4-h sampling period on pnd 36. Note that the number of LH pulses in the 4-h sampling period in control rats significantly increased between pnd 34 and 36. (F) Summary showing that the administration of SB222200 significantly reduced the amplitude of LH pulses on both pnd 34 and 36. $^{\#}P < 0.05$ *vs.* aCSF group on the same pnd; $^{*}P < 0.05$ *vs.* aCSF on pnd 34 (n = 6-7 per group).

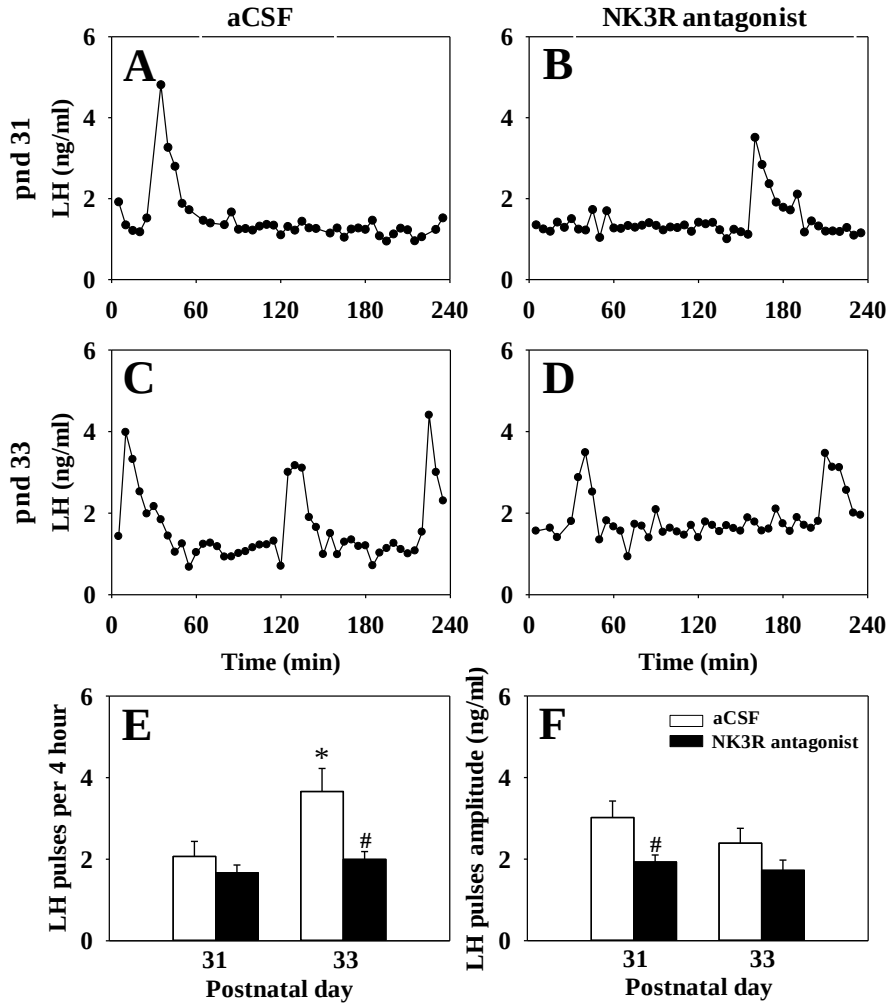

Fig. 3.4 Effects of a NK3R antagonist (SB222200) on LH pulses in prepubertal female rats fed a high fat diet (45% kcal of fat intake) starting from pnd 21. (A, C) Representative examples of LH profiles on pnd 31 and 33 in animals administered aCSF (12 µl/d, icv) as vehicle. (B, D) Representative examples of LH profiles on pnd 31 and 33 in animals administered SB222200 (24 nmol/d, icv). (E) Summary showing that the administration of SB222200 significantly reduced the number of LH pulses in the 4-h sampling period on pnd 33. Note that the number of LH pulses in the 4-h sampling period in control rats significantly increased between pnd 31 and 33. (F) Summary showing that the administration of SB222200 significantly reduced the amplitude of LH pulses in the 4-h sampling period on pnd 31. $^{\#}P < 0.05$ *vs.* aCSF group on the same pnd; $^{*}P < 0.05$ *vs.* aCSF on pnd 31 (n = 7-9 per group).

3.5 Discussion

It is well established from studies in humans with inactivating mutations that NKB-NK3R signalling plays a critical role in pubertal development (Topaloglu *et al.* 2009, Gianetti *et al.* 2010, Francou *et al.* 2011, Tusset *et al.* 2012). The present study further confirms the physiological importance of endogenous NKB-NK3R signalling for the initiation of puberty in an animal model by showing that the timing of puberty is profoundly delayed in prepubertal female rats receiving continuous chronic central administration of SB222200, a selective NK3R antagonist. Furthermore, we have demonstrated a role for NKB-NK3R signalling in precocious puberty consequent to an obesogenic diet by showing that the advanced puberty onset in female rats fed with a high fat diet is also delayed by continuous administration of SB222200. Our findings are consistent with, and extend, recent studies (Gill *et al.* 2012, Navarro *et al.* 2012, Nakahara *et al.* 2013) that have shown that peripheral daily administration of SB222200 caused a modest, non-significant delay of puberty in female mice (Gill *et al.* 2012). Although the ability of SB222200 to cross the blood-brain barrier was tested in that study, the lack of significant developmental timing delay may be due to the limited duration of blockade produced by daily mode of administration (Gill *et al.* 2012). In the same vein, this rationale could also apply to the aforementioned study by Navarro *et al*. that intermittent central administration of SB222200 only induced a modest delay in timing of puberty (Navarro *et al.* 2012). In this sense, a study published recently by Nakahara *et al.*

showed that continuous peripheral administration (via osmotic mini-pump) of senktide, a NK3R agonist, advances puberty in female rats (Nakahara *et al.* 2013); adding further support to the critical role of NKB-NK3R signalling in initiating the puberty onset.

Reproduction is an energy-demanding process which is mediated by metabolic cues that eventually contribute to the regulation of GnRH release. Positive energy balance leads to precocious puberty onset while negative energy balance delays the timing of puberty onset. The present study demonstrates for the first time that a NK3R antagonist retard precocious puberty in overfed female rats, which would appear to be in agreement with the observation that chronic administration of a NK3R agonist partially reversed the pubertal failure in underfed prepubertal female rats (Navarro *et al.* 2012). *Tac2* mRNA expression in the ARC is up-regulated in overfed rats with advanced puberty (Li *et al.* 2012), whereas *Tac2* mRNA expression is down-regulated in the ARC of underfed rats with delayed puberty (Navarro *et al.* 2012). Thus, NKB gene expression is subjected to modulation of metabolic cues, which is in keeping with the notion that NKB may be a prepubertal signalling system of the metabolic status (Navarro 2013). Collectively, these studies support the idea that NKB-NK3R signalling plays a pivotal role in pubertal development and the altered timing of puberty, irrespective of nutritional status. However, a major conundrum regarding this role of NKB-NK3R signalling arose due to the recent finding that genetic *Tacr3* knockout mice achieve a normal timing of sexual maturation,

although they presented with lower uterine weight, abnormal oestrous cyclicity and subfertility (Yang *et al.* 2012). It is possible these *Tacr3* null mice retain residual NK3R activity, or in the absence of NK3R, NKB activates other tachykinin receptors to stimulate GnRH release (Steiner and Navarro 2012, de Croft *et al.* 2013).

It is well established that puberty onset is associated with an acceleration of LH pulse frequency (Sisk and Foster 2004, Plant 2008, Mayer *et al.* 2010, Li *et al.* 2012). It has been shown in the present study that pubertal delay in response to NK3R antagonism was accompanied by a reduction in LH pulse frequency and amplitude in both normal and high fat diet fed rats. Before the onset of puberty there is an up-regulation of ARC *Tac2* and *Tacr3* mRNA expression (Gill *et al.* 2012, Navarro *et al.* 2012), which suggests potential involvement of NKB-NK3R signalling in the acceleration of the GnRH pulse generator during this critical developmental stage. In the present study, we demonstrate for the first time that NK3R antagonism delays the increase in LH pulse frequency during pubertal development in female rats. This is consistent with studies showing that chronic administration of SB222200 tends to decrease basal LH levels (Navarro *et al.* 2012), and chronic administration of the NK3R agonist, senktide, tends to increase the frequency of pulsatile LH release in prepubertal female rats (Nakahara *et al.* 2013). Additionally, acute injections of senktide induced LH pulses in prepubertal macaques (Ramaswamy *et al.* 2011), rats (Grachev *et al.* 2012b) and sheep (Nestor *et al.* 2012). In the present study,

NK3R antagonism was also shown to reduce the amplitude of LH pulses; however, the mechanisms underlying this are not known. Interestingly, this phenomenon is reminiscent of the LH profiles in patients with *TAC3* or *TACR3* mutations that the LH pulse frequency and amplitude is both significantly reduced (Francou *et al.* 2011, Young *et al.* 2013). Furthermore, central administration of NKB increased the frequency and amplitude of MUA volleys in the ARC (mirroring the LH pulses) in goat, which would appear to be in keeping with the present findings (Wakabayashi *et al.* 2010). Thus, NKB-NK3R signalling may involve not only in the regulation of the LH pulse frequency, but also LH pulse amplitude, although the underlying mechanisms remain to be investigated. Furthermore, we have previously shown that precocious puberty associated with over-nutrition is accompanied by an earlier onset of accelerated LH pulse frequency and concomitant up-regulation of ARC *Tac2* mRNA expression (Li *et al.* 2012). The results from the present study suggest that endogenous NKB-NK3R signalling may underlie the acceleration of LH pulse frequency associated with precocious puberty in overfed rats.

The neural mechanisms underlying modulation of LH pulse frequency by NKB-NK3R signalling is unclear, particularly because the neural construct comprising the GnRH pulse generator *per se* remains enigmatic. There is increasing evidence that the network of KNDy neurones in the ARC may represent a significant component of the GnRH pulse generator (Lehman *et al.* 2010, Wakabayashi *et al.* 2010). It is speculated that reciprocal stimulatory and

inhibitory interactions between KNDy neurones mediated by NKB and Dyn, respectively, result in pulsatile Kiss1 release to drive pulsatile release of GnRH (Navarro *et al.* 2009a, Lehman *et al.* 2010, Wakabayashi *et al.* 2010). In studies supporting this mode of action, Goodman *et al*. (Goodman *et al.* 2013) recently demonstrated that intra-ARC administration of SB222200 or the selective κ-opioid receptor antagonist, nor-BNI, decreased or increased LH pulse frequency, respectively, in the ovariectomised ewe. Although the failure of NK3R antagonism to reduce LH pulse frequency in the ovariectomised rat (Grachev *et al.* 2012a) remains an unexplained and unexpected observation, the activation of this receptor in the gonadal intact adult or prepubertal rat evokes LH pulses (Navarro *et al.* 2011a, Grachev *et al.* 2012b, Kinsey-Jones *et al.* 2012). Thus, it is reasonable to suggest that the suppression of LH pulses and pubertal delay observed in the present study may reflect antagonism of the ARC population of NK3R; more specifically, the NK3R expressed on KNDy neurones. It is speculated that NKB act on ARC KNDy neurones to release Kiss1 which, in turn, act on Kiss1r expressed GnRH neurones (Navarro 2013). This speculation is supported by various evidence including: (a) senktide-induced pulsatile LH secretion is dependent on Kiss1r (Ramaswamy *et al.* 2011, Garcia-Galiano *et al.* 2012b, Grachev *et al.* 2012b); (b) isolated mouse GnRH neurones are insensitive to NKB, while Kiss1 neurones are robustly activated and reversed by NKB antagonist (Navarro *et al.* 2011a, Kirilov *et al.* 2013, Ruka *et al.* 2013); (c) GnRH neurone-specific deletion of Kiss1r is associated with a failure to go

through puberty and infertility (Kirilov *et al.* 2013). Together, these data strongly suggest an action of NKB upon KNDy neurones; however, we cannot rule out any additional action of NKB on other brain areas since: (a) it has been reported that NKB causes GnRH secretion in GT1-7 GnRH neurones (Glidewell-Kenney *et al.* 2013); (b) earlier morphologic evidence indicated that NKB could influence GnRH secretion at the level of the median eminence via interaction with NK3R on GnRH terminal fibres (Krajewski *et al.* 2005); (c) activation of NK3R at the level of GnRH terminals in the median eminence stimulates GnRH release and this effect is independent of Kiss1 (Gaskins *et al.* 2013). Further research is necessary to establish the precise site and mechanism by which NKB-NK3R signalling regulates pulsatile GnRH secretion.

In conclusion, the present study demonstrates that endogenous NKB-NK3R signalling plays a role in controlling the timing of puberty and the associated changes in GnRH pulse generator frequency in normal and overfed female rats.

CHAPTER FOUR: TRIPHASIC EFFECTS OF NKB AND NMDA ON LH SECRETION IN FEMALE RATS

4.1 Introduction

GnRH neurones, located within the hypothalamus, form the final common pathway for the control of the reproductive system. GnRH neurones secrete GnRH to prompt the release of LH and FSH from the anterior pituitary. This process is influenced by several factors, including neuropeptides (e.g. Kiss1 and NKB), neurotransmitters (e.g. glutamate) and sex steroids (e.g. E_2). A close interplay exists among those factors allowing fine control of GnRH release.

Recent discoveries of Kiss1-Kiss1r and NKB-NK3R signalling have extensively expanded our knowledge of hypothalamic control of GnRH release (de Roux *et al.* 2003, Topaloglu *et al.* 2009). A plethora of studies have indicated Kiss1 as a predominant stimulatory neuropeptide on GnRH/LH secretion regardless of the sex steroids conditions (Roa *et al.* 2006, Pielecka-Fortuna *et al.* 2008, Navarro *et al.* 2009b). In contrast, stimulatory, inhibitory, or even null effects of the agonist of NK3R, senktide, on LH secretion have been reported: central administration of senktide stimulates LH release in intact rodents (Navarro *et al.* 2011b, Kinsey-Jones *et al.* 2012), castrated male monkeys (Ramaswamy *et al.* 2010) and intact female sheep (Billings *et al.* 2010). Other studies have shown the opposite effects, with senktide decreasing the circulating LH levels in OVX rodents (Sandoval-Guzman and Rance 2004, Navarro *et al.* 2009a, Navarro *et al.*

2011a), and suppressing pulsatile LH release in OVX goats and OVX rats (Wakabayashi *et al.* 2010, Kinsey-Jones *et al.* 2012, Grachev *et al.* 2014a). Interestingly, no effect on LH secretion was shown in other studies in OVX mice primed with E_2 and in intact male rats (Navarro *et al.* 2009a, Corander *et al.* 2010). The discrepancies among the above studies might be due to the differences in the species, sex and gonadal status. Indeed, an ensuing study regarding the sex differences has shown that NKB stimulated LH release in a sexually dimorphic fashion: NKB stimulated LH release in female rats, regardless of the stage of development, but failed to stimulate LH release in male rats after puberty onset, and this phenomenon was independent of the sex steroid milieu (Ruiz-Pino *et al.* 2012). This finding helped to resolve a small part of the above discrepancies. In addition, other studies addressing the gonadal status/sex steroid milieu have yielded complicated results, with some showing the reverse effects of NKB on LH secretion in OVX rats after E_2 treatment, and others displaying the same inhibitory effects with or without E_2 treatment (Navarro *et al.* 2011a, Grachev *et al.* 2012a). The basis underlying these remains to be further investigated.

The neurotransmitter glutamate is another important regulator of GnRH release. Glutamate, acting through the NMDA receptor, a glutamate receptor subtype that is required for GnRH release, plays an important role in many aspects of the reproductive system, including the onset of puberty (Urbanski and Ojeda 1990, Meijs-Roelofs *et al.* 1991), pre-ovulatory LH surge (Brann and Mahesh 1991,

Meijs-Roelofs *et al.* 1991) and the maintenance of pulsatile LH release (Bourguignon *et al.* 1989, Ping *et al.* 1995). Similar to NKB-NK3R signalling, the sex steroid milieu appears to be very important for the LH releasing ability of NMDA, an agonist of NMDA receptor. NMDA has either null or inhibitory effects on LH secretion in OVX animals without exogenous E_2 treatment (Estienne *et al.* 1990, Reyes *et al.* 1990, Reyes *et al.* 1991, Brann and Mahesh 1992, Arias *et al.* 1993). In contrast, NMDA stimulates LH secretion in animals with intact gonadal status across various species, which is in keeping with the general excitatory role of glutamate (Schainker and Cicero 1980, Wilson and Knobil 1982, Gay and Plant 1987, Jansen *et al.* 1991, Brann and Mahesh 1992, d'Anglemont de Tassigny *et al.* 2010, Garcia-Galiano *et al.* 2012a). Furthermore, the inhibitory or null effects of NMDA on LH release in OVX rats, sheep and monkeys are shifted to stimulatory effects in the presence of high levels of E_2 (Estienne *et al.* 1990, Reyes *et al.* 1991, Arias *et al.* 1993).

It appears that NMDA increases LH release in the presence of physiological relevant levels of sex steroids when gonads are intact, whereas it inhibits LH release in the presence of low levels of sex steroids when gonads are removed; effects reminiscent of NKB-NK3R signalling described above. A large number of studies have indicated that instead of direct actions on GnRH neurones, NKB acts through other signalling systems such as Kiss1 and Dyn to affect GnRH release (Ramaswamy *et al.* 2011, Garcia-Galiano *et al.* 2012b, Grachev *et al.* 2012b, Kinsey-Jones *et al.* 2012). Similar to NKB, NMDA does not seem to act

on GnRH neurones directly, since either central or peripheral treatment of NMDA failed to induce c-Fos expression in GnRH neurones in rodents (Saitoh *et al.* 1991, Lee *et al.* 1993, d'Anglemont de Tassigny *et al.* 2010). Alternatively, it is suggested that peripheral administration of NMDA could cross the blood-brain barrier to activate ARC Kiss1 neurones since the stimulatory effects of peripheral NMDA on LH release were lost in *Kiss1* knockout mice (d'Anglemont de Tassigny *et al.* 2010). Therefore, the stimulatory effects of NMDA in the presence of high levels of sex steroids may be due to the activation of Kiss1 neurones in the ARC. Furthermore, NKB-NK3R signalling in the ARC exerts an inhibitory effect on pulsatile LH release in OVX rats, which may involve Dyn signalling (Grachev *et al.* 2012a). Thus, it is reasonable to speculate that peripheral administration of NMDA act through KNDy neurones to affect LH release, that is, via Kiss1-Kiss1r signalling to stimulate LH release or via NKB-NK3R signalling to inhibit LH release, depending on the prevailing sex steroids milieu.

4.2 Aims and objectives

To test the hypothesis that the effects of NKB-NK3R and NMDA signalling systems on LH release in OVX rats are dependent on the circulating levels of E_2 and that the different effects of NMDA on LH release are mediated via the KNDy signalling systems.

a) To determine the effects of senktide or NMDA on LH release in OVX rats in the presence of different levels of E_2 replacement.

b) To establish whether the suppressive effect of NMDA on LH release in low E_2 condition is through NKB-NK3R signalling.

c) To establish whether the stimulatory effect of NMDA on LH release in high E_2 condition is through Kiss1-Kiss1r signalling.

4.3 Materials and methods

4.3.1 Animals and surgical procedures

Under general anaesthesia, adult Sprague-Dawley rats were OVX. In addition, animals receiving E_2 replacement were implanted subcutaneously with either one or two Silastic^{TM} E_2 capsules, while animals without E_2 replacement were replaced with two Silastic^{TM} capsules filled with arachis oil (see section 2.2). Meanwhile, animals were also implanted with unilateral icv guide cannulae directed towards the left lateral cerebral ventricle (see section 2.2.2.4). After a 10-d recovery period, rats were fitted with two indwelling cardiac catheters via the jugular vein, as described in section 2.2.2.6. Experimentation commenced 3 d later (Fig. 4.1).

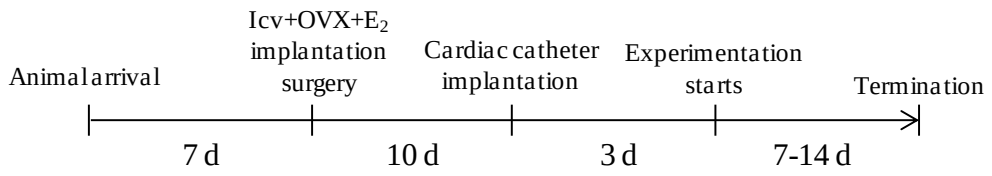

Fig. 4.1 Timeline diagram of various surgical and experimental procedures for investigating the effects of NKB-NK3R and NMDA signalling on LH release in OVX rats replaced with different levels of E_2.

4.3.2 Experiment 1: Effects of senktide on LH secretion in OVX rats replaced with different levels of E_2

An icv injection cannula (Plastics One) with extension tubing preloaded with senktide (Tocris Bioscience) was inserted into the guide cannula, with 1 µl of air separating the fluid in the tubing from the lateral ventricle system before the actual injection. The tip of injection cannula extended 1 mm beyond the guide cannula to reach the ventricle. The distal end of the tubing was extended outside of the cage and connected to a 25 µl Hamilton syringe (Waters International), thereby allowing remote infusion without disturbing the rat during the experiment (see section 2.2.3.3). Rats were also attached via one of the two cardiac catheters to a computer-controlled automated blood sampling system, which allows for the intermittent withdrawal of small blood samples (see section 2.2.3.1). Once connected, animals were left undisturbed for 1 h before sampling commenced between 1000 and 1100 h; samples were collected every 5 min for 6

h. At the end of the experiment, 150 µl blood was withdrawn from the cardiac catheter and centrifuged at 800 rpm for 15 min in a refrigerated centrifuge. The plasma was aspirated and stored in a -20°C freezer for later assay to determine E_2 concentration.

To examine the effects of senktide on LH secretion in the presence of different E_2 levels, senktide (600 pmol in 4 µl aCSF) was administered (icv) over 5 min after 2-h controlled blood sampling into OVX rats without E_2 replacement (OVX+0×E_2, n = 7), into OVX rats implanted with one E_2 capsule (OVX+1×E_2, n = 10), and into OVX rats implanted with two E_2 capsules (OVX+2×E_2, n = 8). The dose of senktide was adopted from Kinsey-Jones *et al.* (2012).

4.3.3 Experiment 2: Effects of NMDA on LH secretion in OVX rats replaced with different levels of E_2

On the morning of experimentation, the rats were attached to the automated blood sampling system, as described above. After 2 h of controlled blood sampling, NMDA (30 mg/kg in 0.2 ml saline, Sigma-Aldrich) was infused (iv) via one of the cardiac catheters over the period of 5 min into OVX+0×E_2 rats (n = 6), into OVX+1×E_2 rats (n = 8), and into OVX+2×E_2 rats (n = 8). The dose of NMDA was adopted from previous studies (Carbone *et al.* 1992, Arias *et al.* 1993).

4.3.4 Experiment 3: Effects of NMDA on LH secretion in OVX+0×E_2 rats in the presence of a NK3R antagonist

On the morning of experimentation, OVX+0×E_2 rats were attached to the automated blood sampling system and equipped with the icv injection system, as described above. After 1 h 45 min of blood sampling, SB222200 (15 nmol in 4 µl aCSF/15% DMSO/20% cyclodextrin, n = 7, Tocris Bioscience), a NK3R antagonist (Sarau *et al.* 2000), or aCSF/15% DMSO/20% cyclodextrin (4 µl, n = 6) was administered (icv) over 5 min into OVX+0×E_2 rats. 15 min later, NMDA (30 mg/kg) was infused (iv) via one of the cardiac catheters over 5 min. Additional controls were administered (icv) with SB222200, followed by iv infusion of saline 15 min later (n = 6). The dose of SB222200 was deduced from one of our previous studies with intra-ARC administration of 0.5 nmol SB222200 (Grachev *et al.* 2012a). A preliminary dose (5 nmol SB222200) failed to block the inhibitory effects of NMDA on LH release (data not shown), which prompted us to adopt a higher dose of 15 nmol.

4.3.5 Experiment 4: Effects of NMDA on LH secretion in OVX+2×E_2 rats in the presence of a Kiss1r antagonist

OVX+2×E_2 rats were attached to the automated blood sampling system and equipped with the icv injection system on the morning of experimentation, as described above. After 1 h 45 min of blood sampling, peptide-234 (3 nmol in 4

µl aCSF; n = 5, Sigma-Aldrich), a Kiss1r antagonist (Roseweir *et al.* 2009), or aCSF (4 µl, n = 5) was infused (icv) into the rats. Fifteen minutes later, NMDA (30 mg/kg) was infused (iv) via one of the cardiac catheters. Additional controls were administered (icv) with peptide-234, followed by iv infusion of saline 15 min later (n = 4). The dose of peptide-234 was adopted from Roseweir *et al.* (2009).

4.3.6 Experiment 5: Effects of implantation of different numbers of E_2 capsules on circulating levels of E_2 in OVX rats, comparing to intact female rats

Blood samples for the measurement of plasma E_2 were obtained as described above (n = 7, 8, 8, for OVX+0×E_2, OVX+1×E_2 and OVX+2×E_2 groups, respectively). An additional group of ovary-intact Sprague-Dawley rats from Harlan Laboratories (Oxfordshire, UK) were monitored daily for normal ovarian cyclicity by means of vaginal cytology. Rats with normal ovarian cyclicity (n = 4) were implanted with cardiac catheters to enable collection of blood samples (150 µl) for measurement of E_2 levels at oestrus and proestrus stages that would be used respectively as physiological low and high E_2 controls. Blood samples (150 µl) were taken at 0900 h on proestrus and 1800 h on oestrus since E_2 levels reach peak and nadir around these times respectively (Smith *et al.* 1975, Nequin *et al.* 1979). Normal ovarian cyclicity in those studies was defined as having at least 2

consecutive normal cycles, which last for 4 d with 1 d of oestrus. Rats with 5 d of cycles were excluded since they had a different fluctuation dynamic of E_2 levels (Nequin *et al.* 1979).

A double-antibody radioimmunoassay (ImmuChem; MP Biomedicals, Orangeburg, NY) was used to estimate the E_2 content of the plasma samples (50 µl) following the manufacturer's protocol. The sensitivity of the assay was 7.2 pg/ml. The intraassay variation was 9.6%, and the interassay variation was 10.1%.

5.3.4 Statistical analysis

The effect of pharmacological agents on LH secretion was calculated by comparing the area under the LH profile [area under the curve (AUC); representing the total amount of LH secretion within a certain period] in the 1st and 2nd 1-h post-treatment period with that in the 1-h pre-treatment period, using SigmaPlot version 11 (Systat Software, San Jose, CA). LH pulses in experiment 3 were established by use of the algorithm ULTRA (Van Cauter 1988). The effects of pharmacological agents on pulsatile LH secretion were analysed by comparing the mean LH pulse interval in the 2-h period preceding treatment with that in two consecutive 2-h post-treatment periods. The duration (min) of the 2-h pre-treatment, and the 1st and 2nd 2-h post-treatment periods, was divided by the number of LH pulses detected in each of these periods to give the appropriate LH pulse interval. The effect of the baseline of LH secretion in

different levels of E_2 was calculated by comparing the AUC in the 1-h pre-treatment period. E_2 levels in OVX+0×E_2, OVX+1×E_2 and OVX+2×E_2 were calculated from means of E_2 levels detected 10 d after OVX. E_2 levels in oestrus or proestrus rats were calculated from means detected during oestrus or proestrus phases in three consecutive oestrous cycles. Statistical significance was tested using one-way ANOVA followed by Dunnett's test. All data were shown as mean ± S.E.M. $P < 0.05$ was considered statistically significant.

4.4 Results

4.4.1 Experiment 1: Effects of senktide on LH secretion in OVX rats replaced with different levels of E_2

To investigate the effects of NKB on LH secretion in different E_2 levels, we examined the effects of centrally administered senktide, a NK3R agonist, on LH secretion in OVX rats replaced with different numbers of E_2 capsules. Central administration of senktide inhibited LH secretion in OVX+0×E_2 rats (AUC in 1-h pre-injection *vs*. AUC in 2nd 1-h post-injection: 283.5 ± 23.3 *vs*. 188.0 ± 13.9 ng/ml.min; $P < 0.05$) (Fig. 4.2). In contrast, senktide briefly stimulated LH secretion in OVX+2×E_2 rats (AUC in 1-h pre-injection *vs*. AUC in 1st 1-h post-injection: 169.6 ± 6.0 *vs*. 207.3 ± 15.0 ng/ml.min; $P < 0.05$) and this stimulation effect was not seen in the second h (AUC in 1-h pre-injection *vs*. AUC in 2nd 1-h post-injection: 169.6 ± 6.0 *vs*. 146.8 ± 7.9 ng/ml.min; $P > 0.05$)

(Fig. 4.2). Most interestingly, senktide had no effects on LH secretion in OVX+1×E_2 rats (AUC in 1-h pre-injection *vs*. AUC in 1st 1-h post-injection *vs*. AUC in 2nd 1-h post-injection: 216.6 ± 19.7 *vs*. 229.4 ± 20.6 *vs*. 218.4 ± 22.3 ng/ml.min; $P > 0.05$) (Fig. 4.2). Of note, the baseline of LH secretion was significantly inhibited in the E_2 replaced rats (AUC in 1-h pre-injection, OVX+0×E_2 *vs*. OVX+1×E_2 *vs*. OVX+2×E_2: 283.5 ± 23.2 *vs*. 216.6 ± 19.7 *vs*. 169.6 ± 6.0 ng/ml.min; $P < 0.05$).

4.4.2 Experiment 2: Effects of NMDA on LH secretion in OVX rats replaced with different levels of E_2

To investigate the effects of NMDA on LH secretion in different E_2 levels, we examined the effects of peripherally infused NMDA, a NMDA receptor agonist, on LH secretion in OVX rats replaced with different numbers of E_2 capsules. Peripheral infusion of NMDA inhibited LH secretion in OVX+0×E_2 rats (AUC in 1-h pre-injection *vs*. AUC in 2nd 1-h post-injection: 285.0 ± 8.7 *vs*. 232.2 ± 10.6 ng/ml.min; $P < 0.05$) (Fig. 4.3). In contrast, NMDA briefly stimulated LH secretion in OVX+2×E_2 rats (AUC in 1-h pre-injection *vs*. AUC in 1st 1-h post-injection: 163.8 ± 16.4 *vs*. 222.1 ± 17.6 ng/ml.min; $P < 0.05$) and this stimulation effect was not seen in the second h (AUC in 1-h pre-injection *vs*. AUC in 2nd 1-h post-injection: 163.8 ± 16.4 *vs*. 146.3 ± 17.5 ng/ml.min; $P < 0.05$) (Fig. 4.3). NMDA had no effects on LH secretion in OVX+1×E_2 rats similar to

that of senktide (AUC in 1-h pre-injection *vs*. AUC in 1st 1-h post-injection *vs*. AUC in 2nd 1-h post-injection: 231.3 ± 23.5 *vs*. 236.8 ± 29.6 *vs*. 203.8 ± 29.5 ng/ml.min; $P > 0.05$) (Fig. 4.3). The baseline of LH secretion was significantly inhibited in E_2 capsules implanted rats as described before (AUC in 1-h pre-injection, OVX+0×E_2 *vs*. OVX+1×E_2 *vs*. OVX+2×E_2: 285.0 ± 8.7 *vs*. 231.3 ± 23.5 *vs*. 163.8 ± 16.4 ng/ml.min; $P < 0.05$).

4.4.3 Experiment 3: Effects of NMDA on LH secretion in OVX+0×E_2 rats in the presence of a NK3R antagonist

To investigate whether NMDA-induced LH suppression in OVX+0×E_2 rats was through the NKB-NK3R signalling, we examined the effects of peripherally administered NMDA on LH secretion in the presence of a NK3R receptor antagonist. Peripheral administration of NMDA-induced LH suppression was blocked by pre-treatment of SB222200, a NK3R antagonist (AUC in 2nd 1-h post-injection, vehicle + NMDA group *vs.* SB222200 + NMDA group: 228.9 ± 7.5 *vs*. 262.4 ± 12.5 ng/ml.min; $P < 0.05$) (Fig. 4.4). Administration of aCSF (icv) with NMDA (iv) inhibited the LH secretion similar to the LH response of NMDA alone, whereas administration of SB222200 (icv) with saline (iv) had no effects on LH secretion (Fig. 4.4). Furthermore, peripheral administration of NMDA suppressed the pulsatile release of LH (LH pulse interval, 2-h pre-injection *vs.* 1st 2-h post-injection: 25.0 ± 2.1 *vs*. 37.8 ± 1.8 min; $P < 0.05$).

This suppression was blocked by co-administration of SB222200 (LH pulse interval in 1st 2-h post-injection, vehicle + NMDA *vs.* SB222200 + NMDA: 37.8 ± 1.8 *vs*. 30.8 ± 1.5 min; $P < 0.05$). SB222200 co-administered with saline had no effects on pulsatile LH release.

4.4.4 Experiment 4: Effects of NMDA on LH secretion in OVX+2×E_2 rats in the presence of a Kiss1r antagonist

To investigate whether NMDA-induced LH release in OVX+2×E_2 rats was through the Kiss1-Kiss1r signalling, we examined the effects of peripherally administered NMDA on LH secretion in the presence of a Kiss1r antagonist. Peripheral administration of NMDA-induced LH release was blocked by pre-treatment of peptide-234, a Kiss1r antagonist (AUC in 2nd 1-h post-injection, vehicle + NMDA group *vs.* peptide-234 + NMDA group: 218.8 ± 12.2 *vs*. 172.7 ± 15.0 ng/ml.min; $P < 0.05$) (Fig. 4.5). Administration of aCSF (icv) with NMDA (iv) stimulated the LH secretion similar to the response of NMDA alone, while administration of peptide-234 (icv) with saline (iv) had no effects on LH secretion (Fig. 4.5).

4.4.5 Experiment 5: Effects of implantation of different numbers of E_2 capsules on circulating levels of E_2 in OVX rats, comparing with intact female rats

The circulating levels of E_2 were significantly increased in OVX rats implanted with one E_2 capsule compared with that without E_2 capsule (OVX+0×E_2 *vs*. OVX+1×E_2: 12.7 ± 1.5 *vs*. 23.8 ± 2.0 pg/ml; $P < 0.05$) (Fig. 4.6). The E_2 levels were further increased in OVX rats implanted with two E_2 capsules (OVX+1×E_2 *vs*. OVX+2×E_2: 23.8 ± 2.0 *vs*. 36.8 ± 2.4 pg/ml; $P < 0.05$) (Fig. 4.6). Compared with the physiological controls, the circulating levels of E_2 in OVX+2×E_2 rats were not different to that in intact female rats in oestrus stage (36.8 ± 2.4 *vs*. 45.2 ± 6.6; $P > 0.05$) but significant lower to that in proestrus stage (36.8 ± 2.4 *vs*. 96.4 ± 6.9; $P < 0.05$) (Fig. 4.6). Furthermore, the E_2 levels in both OVX+0×E_2 and OVX+1×E_2 rats were lower to that in oestrus stage ($P < 0.05$) (Fig. 4.6).

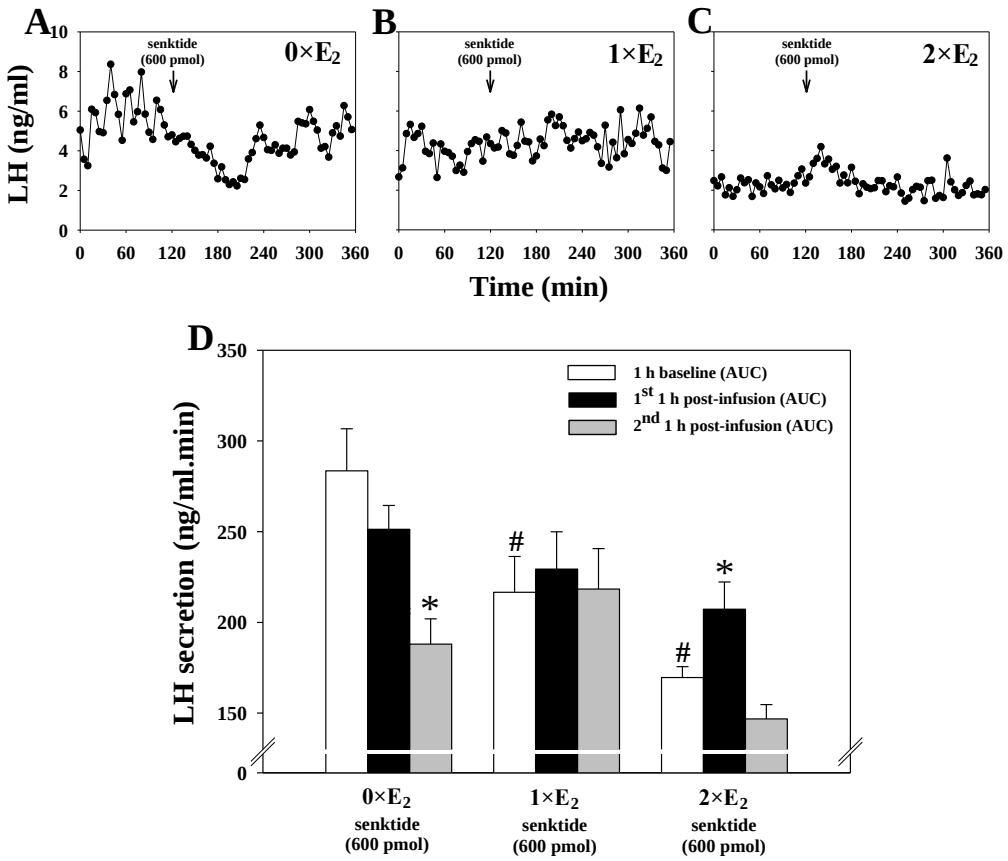

Fig. 4.2 Effects of senktide on LH secretion in OVX rats replaced with different levels of E_2. Representative LH profiles illustrating the effect of icv administration (arrow) of senktide (600 pmol) on LH secretion in OVX rats without E_2 replacement (labelled as $0\times E_2$) (A), with one E_2 capsule replacement (labelled as $1\times E_2$) (B), or with two E_2 capsules replacement (labelled as $2\times E_2$) (C). Central administration of senktide inhibited LH secretion in OVX+$0\times E_2$ rats, but stimulated LH secretion in OVX+$2\times E_2$ rats and had no effects on LH secretion in OVX+$1\times E_2$ rats, as summarised in D. *$P < 0.05$ *vs.* 1 h baseline within the same group; $^{\#}P < 0.05$ *vs.* 1-h baseline within the $0\times E_2$ group (n = 7-10 per group).

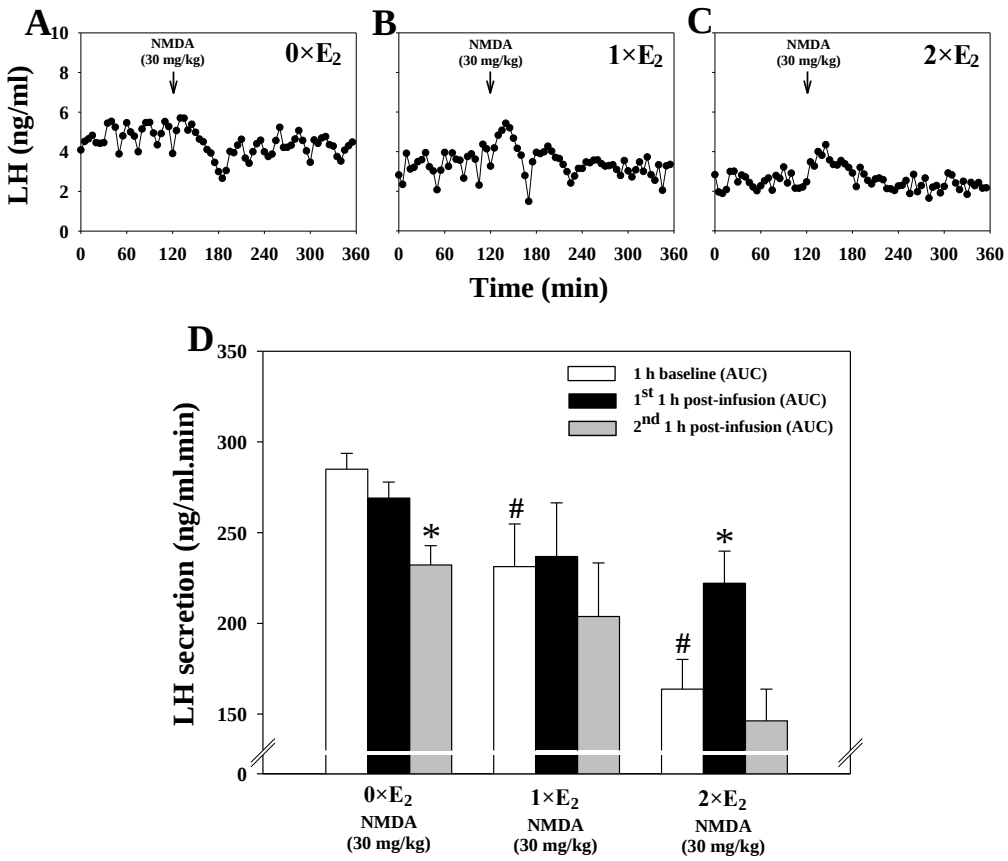

Fig. 4.3 Effects of NMDA on LH secretion in OVX rats replaced with different levels of E_2. Representative LH profiles illustrating the effect of iv infusion (arrow) of NMDA (30 mg/kg) on LH secretion in OVX rats without E_2 replacement (labelled as $0\times E_2$) (A), with one E_2 capsule replacement (labelled as $1\times E_2$) (B), or with two E_2 capsules replacement (labelled as $2\times E_2$) (C). Peripheral administration of NMDA inhibited LH secretion in OVX+$0\times E_2$ rats, but stimulated LH secretion in OVX+$2\times E_2$ rats and had no effects on LH secretion in OVX+$1\times E_2$ rats, as summarised in D. *$P < 0.05$ *vs.* 1-h baseline within the same group; $^{\#}P < 0.05$ *vs.* 1-h baseline within the $0\times E_2$ group (n = 6-8 per group).

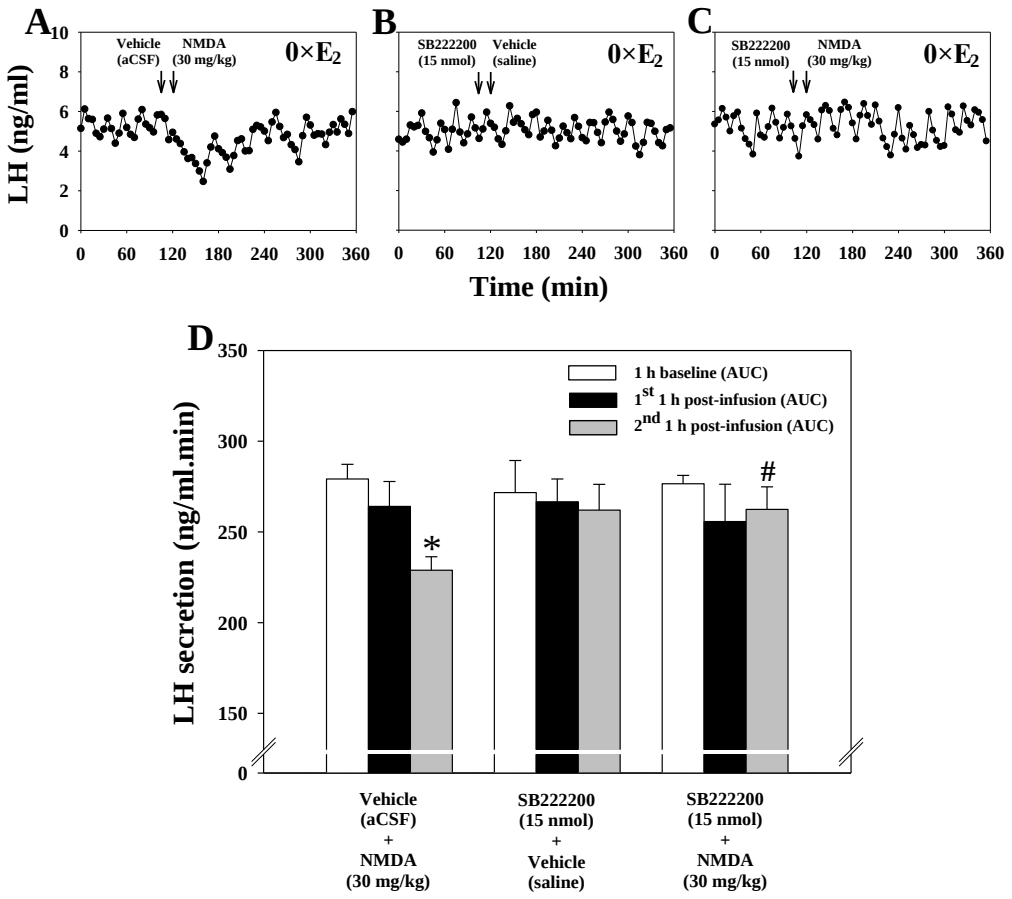

Fig. 4.4 Effects of NMDA on LH secretion in OVX+0×E_2 rats in the presence of a NK3R antagonist. Representative LH profiles illustrating the effect of iv infusion (arrow) of NMDA (30 mg/kg) on LH secretion in OVX+0×E_2 rats in the presence (C) or absence (A) of icv administration (arrow) of SB222200 (15 nmol), a NK3R antagonist, as well as the icv administration of SB222200 (15 nmol) with iv infusion of vehicle (saline) (B). NMDA-induced inhibition of LH secretion in OVX +0×E_2 rats was blocked by pre-treatment of SB222200, a NK3R antagonist, as summarised in D; SB222200 *per se* had no effects on LH secretion. *P < 0.05 *vs.* 1-h baseline within the same group; #P < 0.05 *vs.* the same 1-h period within the group treated with vehicle and NMDA (n = 6-7 per group).

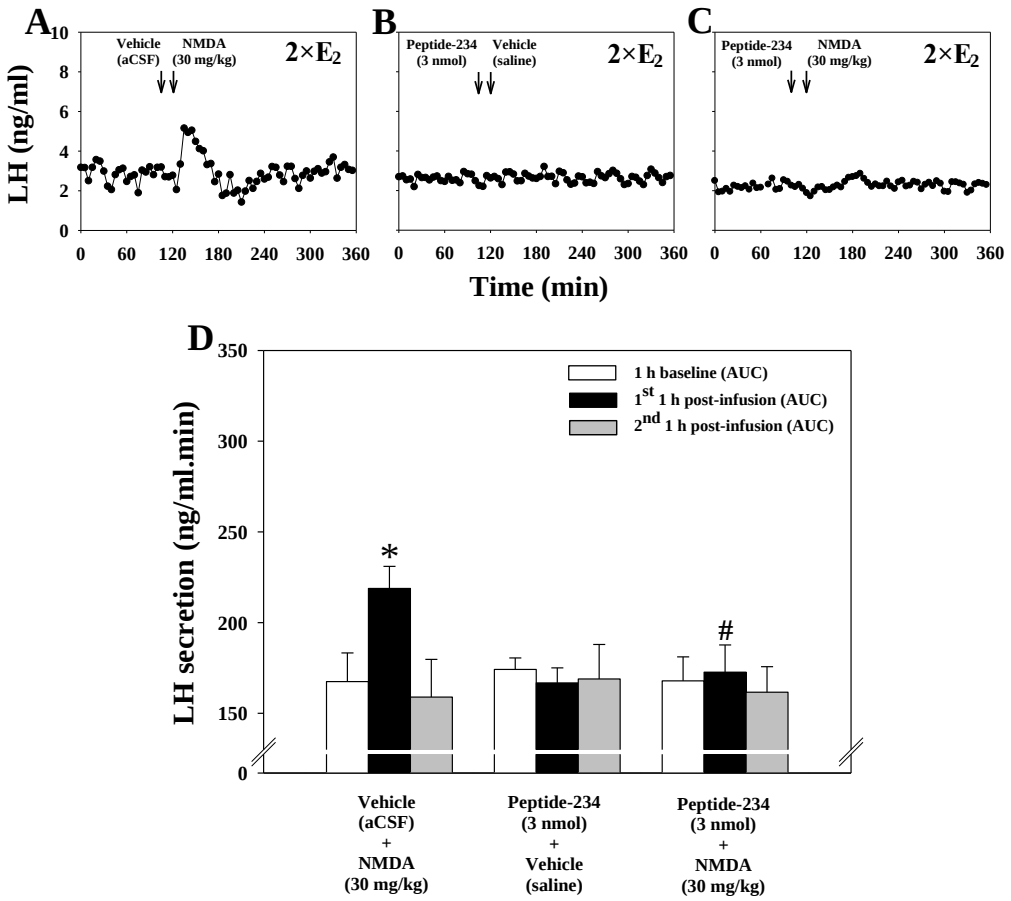

Fig. 4.5 Effects of NMDA on LH secretion in OVX+2×E_2 rats in the presence of a Kiss1r antagonist. Representative LH profiles illustrating the effect of iv infusion (arrow) of NMDA (30 mg/kg) on LH secretion in OVX+2×E_2 rats in the presence (C) or absence (A) of icv administration (arrow) of peptide-234 (3 nmol), a Kiss1r antagonist, as well as the icv administration of peptide (3 nmol) with iv infusion of vehicle (saline) (B). NMDA-induced stimulation of LH secretion in OVX +2×E_2 rats was blocked by pre-treatment of peptide-234, a Kiss1r antagonist, as summarised in D; Peptide-234 *per se* had no effects on LH secretion. *P < 0.05 *vs.* 1 h baseline within the same group; #P < 0.05 *vs.* the same 1-h period within the group treated with vehicle and NMDA (n = 4-5 per group).

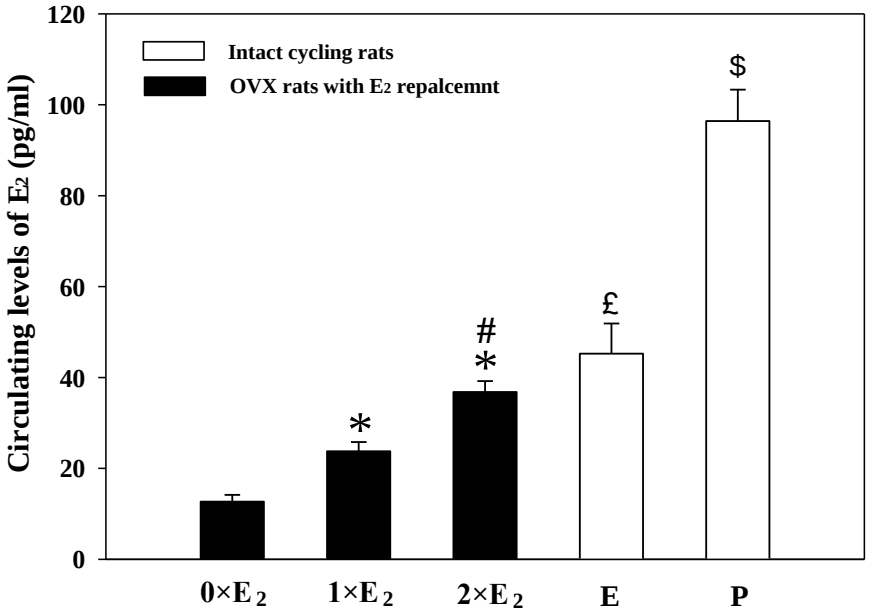

Fig. 4.6 Effects of implantation of different numbers of E_2 capsules on circulating levels of E_2 in OVX rats, comparing to intact female rats. The circulating levels of E_2 were increased in OVX rats implanted with one E_2 capsule (labelled as $1\times E_2$) compared with that in OVX rats without E_2 capsule implantation (labelled as $0\times E_2$). The E_2 levels were further increased in OVX rats replaced with two E_2 capsules (labelled as $2\times E_2$) compared with one capsule. Furthermore, the circulating levels of E_2 in OVX+$2\times E_2$ rats were lower to that in intact female rats in proestrus phase (labelled as P) but not different to that in oestrus phase (labelled as E). However, the E_2 levels in both OVX+$0\times E_2$ and OVX+$1\times$ E_2 rats were lower to that in oestrus phase. *$P < 0.05$ *vs*. $0\times E_2$ group; $^{\#}P < 0.05$ *vs*. $1\times E_2$ group; $^{£}P < 0.05$ *vs*. $1\times E_2$ group, as well as $0\times E_2$ group; $^{\$}P < 0.05$ *vs*. $2\times E_2$ group, as well as E group (n = 4-8 per group).

4.5 Discussion

The results of this study have demonstrated that central administration of a selective NK3R agonist potently suppressed LH release in conditions of low levels of E_2 (OVX without E_2 replacement), but stimulated LH release in conditions of relatively high levels of E_2 (equivalent to oestrus phase in normal cycling rats). Furthermore, NK3R agonist had no effects on LH release in animals with medium circulating levels of E_2 (OVX with one E_2 capsule replacement). These results support and extend previous studies that showed that senktide had a biphasic effects on LH release (Wakabayashi *et al.* 2010, Navarro *et al.* 2011a), adding that an intermediary state may exist between those two phases, given a condition with appropriate E_2 levels. Admittedly, the differences of circulating E_2 levels in those three replacement regimes in the present study were very subtle (Fig. 4.6). However, an extensive difference in LH releasing ability was induced by this comparably narrow range of E_2 levels which might provide an explanation for previous studies showing suppressive, null or stimulatory effects of senktide on LH release in OVX rodents with or without E_2 replacement (Sandoval-Guzman and Rance 2004, Navarro *et al.* 2009a, Navarro *et al.* 2011a, Kinsey-Jones *et al.* 2012). Previous studies from our lab failed to observe the null or stimulatory effects of senktide on LH release using a similar methodology (Grachev *et al.* 2012a, Kinsey-Jones *et al.* 2012). This may be due to the seemingly less potent E_2 replacement in those studies, with intact pulsatile LH release regardless of the E_2 replacement regime (Grachev *et al.* 2012a,

Kinsey-Jones *et al.* 2012). In the present study, LH pulses were presented in the low E_2 replacement regime, whereas they were markedly attenuated in the medium and high E_2 replacement groups, reflecting a stronger E_2 negative feedback control. It should be noted that all of the above E_2 levels were either equal or below the E_2 levels determined in the oestrus phase of the cycle, suggesting that in the rat, the switch from an inhibitory to a stimulatory effect of senktide on LH release happened below the lowest physiological E_2 levels, which is in accordance with the predominant stimulatory role of NKB-NK3R signalling in normal physiological conditions.

In the present study, senktide inhibits LH release in the OVX rats, evidently in the second post-treatment h as calculated by the AUC of LH profiles (Fig. 4.2). This is in agreement with a previous study which showed central administration of senktide inhibited LH level in OVX rats 120 min after the treatment onset (Navarro *et al.* 2011a). In addition, senktide inhibits LH pulses in OVX+0×E_2 rats, which is in keeping with previous studies showing prolongation of the LH pulse interval throughout the 2-h post-treatment period (Grachev *et al.* 2012a, Kinsey-Jones *et al.* 2012, Grachev *et al.* 2014a). These inhibitory effects are through activating the NK3R in the ARC, since intra-ARC administration of senktide dose-dependently inhibited the pulsatile release of LH, an effect blocked by pre-treatment of a NK3R antagonist (Grachev *et al.* 2012a). Furthermore, senktide-induced suppression of pulsatile LH release can be blocked by intra-ARC administration of nor-BNI, a κ-opioid receptor antagonist,

indicating that NKB-NK3R signalling in the ARC is mediated via Dyn, the endogenous ligand for the κ-opioid receptor, to activate κ-opioid receptor, and thus suppress the GnRH pulse generator (Grachev *et al.* 2012a). However, it should be noted that κ-opioid receptors are not abundantly expressed in the vicinity of GnRH neurones (Mitchell *et al.* 1997). Therefore, it is hypothesised that Dyn neurones affect GnRH secretion via an unknown signalling system (Grachev *et al.* 2014b). Overall, since KNDy neurones in the ARC co-express NK3R and Dyn, it is postulated that senktide acts through KNDy neurones to suppress the GnRH release (Grachev *et al.* 2014b), at least when E_2 levels are low.

In contrast to its inhibitory effects in low E_2 conditions, senktide stimulated LH release in a relatively high E_2 environment in OVX rats that were implanted with two E_2 capsules (Fig. 4.2). This is in line with a previous study (Navarro *et al.* 2011a) that showed senktide induced LH release in OVX rats replaced with proestrus levels of E_2. A plethora of studies have indicated that NKB-induced LH release is dependent on Kiss1-Kiss1r signalling (Ramaswamy *et al.* 2010, Garcia-Galiano *et al.* 2012b, Grachev *et al.* 2012b). In rodents, the *Kiss1r* knockout mice was devoid of senktide-induced LH release, while Kiss1r antagonism blocked the senktide-induced release of LH in prepubertal rats (Garcia-Galiano *et al.* 2012b, Grachev *et al.* 2012b). In addition, morphological studies have further strengthened this argument. It was shown that KNDy neurones were interconnected with each other bilaterally in the ARC through

NK3R (Krajewski *et al.* 2010). Furthermore, KNDy neurones were shown to project to the mPOA and median eminence in rodents (Lehman *et al.* 2013). Although it is not known whether Kiss1r protein is expressed in GnRH terminals, *Kiss1r* mRNA is identified in GnRH neurones by *in situ* hybridization in rodents (Irwig *et al.* 2004, Han *et al.* 2005). It is therefore postulated that NKB may act through KNDy neurones, which in turn activate GnRH neurones via either GnRH cell bodies in the mPOA or GnRH terminals in the medial eminence (Navarro 2013, Grachev *et al.* 2014b).

Nevertheless, the most intriguing finding of the present study was the null effect of the senktide on LH release in the medium E_2 replacement regime. An absence of LH response to senktide was previously reported in OVX mice primed with E_2 (Navarro *et al.* 2009a). It was argued that the levels of *NK3R* mRNA in the ARC were potently inhibited by the increased E_2 concentration, leading to the lack of effect of senktide on the LH release (Navarro *et al.* 2011a). However, this argument failed to explain the stimulatory effects of senktide in intact female rats with similar levels of *NK3R* mRNA in the ARC (Navarro *et al.* 2011a). Given the paradigm of the present study, the null effect might be due to the opposing inhibitory and stimulatory effects of senktide on LH release, or due to the unresponsive nature of senktide on LH release at that particular E_2 level, therefore, providing an expected transitional stage (i.e. the 'switch'). The mechanisms underlying this switch from inhibitory to stimulatory, dependent on E_2 level, which is in common with many other neuropeptides (Kalra and Kalra

1983) and neurotransmitters, remain to be established.

In the present study, a similar effect of NMDA on LH release as reported for senktide was observed. NMDA is an agonist of NMDA receptor, a subtype for the neurotransmitter glutamate. Peripheral administration of NMDA potently suppressed LH release in the presence of low levels of E_2 (OVX without E_2 replacement), whereas stimulated LH release in the presence of relatively high levels of E_2 (OVX with two E_2 capsules; equivalent to oestrus phase in normal cycling rats). Furthermore, NMDA had no effects on LH release in the presence of intermediary levels of E_2 (OVX with one E_2 capsule). These data are in keeping with previous studies that showed NMDA inhibited or had no effect on LH release in OVX rats and increased LH release in proestrus rats or OVX rats primed with E_2 (Brann and Mahesh 1992, Arias *et al.* 1993). Comparing the effect of NMDA with senktide, the triphasic LH releasing ability and thresholds of E_2 levels for the switch to happen are similar, which may allude to possible common mechanisms and prompted us to investigate the possible correlation between those two signalling systems.

The inhibitory effects of NMDA on LH release in low E_2 conditions have remained unexplained. The suppressive effects on LH release following administration of NMDA in OVX monkeys are accompanied by a concomitant increase of circulating levels of cortisol (Reyes *et al.* 1990). Thus, NMDA may activate corticotrophin-releasing factor (CRF), the core of hypothalamo-pituitary-adrenal (HPA) axis and a hypothalamic inhibitory

regulator on GnRH release (Li *et al.* 2010), to suppress LH release. Indeed, pre-treatment with a CRF antagonist prevents the decrease of LH release in response to NMDA (Reyes *et al.* 1990). However, NMDA was later shown to increase cortisol levels to the same degree in OVX monkeys with or without E_2 treatment (Reyes *et al.* 1991), therefore, making the above argument less compelling. In the present study, we provided an alternative route for NMDA to suppress LH release in low E_2 conditions which is through the NKB-NK3R signalling system, since a NK3R antagonist blocked the suppressive effects of NMDA on LH release in OVX rats. Although it is not clear where NK3R antagonism is acting, it could be speculated that peripheral administration of NMDA could act through ARC KNDy neurones to suppress the LH release via NKB-NK3R signalling, because (a) KNDy neurones receive inputs from non-KNDy glutamatergic fibres arising from other regions (Lehman *et al.* 2013); (b) KNDy neurones in the ARC co-express NKB and NK3R (Lehman *et al.* 2010); and (c) activation of NK3R in the ARC inhibits the LH release in OVX rats and these effects can by blocked by pre-treatment of Dyn antagonism (Grachev *et al.* 2012a). Thus, we proposed a potent link between glutamate → NKB → Dyn signalling systems may underlie the inhibitory effects of NMDA on LH release. Of note, NK3R antagonism *per se* had no effects on LH release, which is in agreement with previous studies (Noritake *et al.* 2011, Grachev *et al.* 2012a). This indicates that endogenous NKB may not be involved in controlling pulsatile LH release in low E_2 conditions.

We have also shown that the stimulatory effects of NMDA on LH release were through Kiss1-Kiss1r signaling, since peptide-234 blocked the NMDA-induced LH secretion in OVX+2×E_2 rats. This is in keeping with the study that showed that peripheral administration of NMDA activates Kiss1-Kiss1r signalling to increase LH release (d'Anglemont de Tassigny *et al.* 2010). It also concurs with an *in vitro* study that showed that Kiss1 increases glutamatergic transmission directly onto GnRH neurones in an E_2 dependent manner (Pielecka-Fortuna and Moenter 2010). However, NMDA does not seem to act on GnRH neurones directly, making the *in vitro* study less compelling in a physiological condition. Our current studies and previous studies support a Kiss1 dependent GnRH/LH release by NMDA, which is supported by the fact that KNDy neurones receive inputs from non-KNDy glutamatergic fibres (Lehman *et al.* 2013) and KNDy neurones activate GnRH neurones to release GnRH (Navarro 2013). However, the present study did not investigate whether the stimulatory effects of NMDA on LH release in OVX+2×E_2 rats are dependent on the NKB-NK3R siganlling system. This is possible since NK3R agonism stimulates LH secretion in the presence of relatively high levels of E_2 and this stimulation is dependent on Kiss1-Kiss1r signalling (Navarro *et al.* 2011a, Garcia-Galiano *et al.* 2012b). Therefore, we cannot rule out the possibility that NKB-NK3R signalling may also mediate the stimulatory action of NMDA on LH relaese.

In summary, we have presented herein a triphasic LH secretory response to senktide, as well as to NMDA, determined by different levels of circulating E_2.

This is reminiscent of the predominant stimulatory effect of the NKB and glutamate signalling systems on the reproductive system in normal physiological conditions, and the inhibitory effect in conditions of low E_2 such as menopause, ovarian failure or ovariectomy. Furthermore, we suggest that peripheral administration of NMDA might act on KNDy neurones to affect LH release, by providing evidences that NMDA activates the Kiss1-Kiss1r or NKB-NK3R signalling systems to stimulate or inhibit LH release respectively, depending on the prevailing E_2 conditions. This might shed light on the mechanisms of many other neurotransmitters or neuropeptides that have presented a similar triphasic effects on LH release.

CHAPTER FIVE: BIPHASIC EFFECTS OF SUBSTANCE P ON LH SECRETION IN FEMALE RATS

5.1 Introduction

Normal reproductive function requires pulsatile GnRH release controlled by the GnRH pulse generator. The precise neural identity of the GnRH pulse generator remains to be clarified. Recently, emerging evidence has alluded to the KNDy neurones in the ARC as a likely candidate for the intrinsic source of the GnRH pulse generator (Lehman *et al.* 2010). Of the neuropeptides in KNDy neurones, NKB belongs to a family of peptides termed tachykinins, which also include NKA and substance P (Almeida *et al.* 2004). Tachykinins bind to three G protein-coupled receptors, NK1R, NK2R and NK3R. Although, each tachykinin binds preferentially to one of the tachykinin receptors (substance P – NK1R, NKA – NK2R, NKB – NK3R), substance P, NKA and NKB can activate any one of the receptors (Almeida *et al.* 2004).

After its identification as a 11-amino acid peptide (Chang *et al.* 1971), substance P has been shown to be involved in pituitary gonadotrophin secretion with numerous effects. Treatments of substance P in a variety of experimental paradigms in laboratory animals have been shown to stimulate (Vijayan and McCann 1979, Arisawa *et al.* 1990, Navarro *et al.* 2015, Simavli *et al.* 2015), inhibit (Vijayan and McCann 1979, Arisawa *et al.* 1990, Kalra *et al.* 1992) or exert no effects (Eckstein *et al.* 1980, Kalra *et al.* 1992, Sahu and Kalra 1992) on

circulating LH levels, with no changes in levels of FSH (Vijayan and McCann 1979, Eckstein *et al.* 1980). More specifically, central administration (icv) of substance P increased tonic LH release in OVX rats/mice with or without E_2 replacement (Vijayan and McCann 1979, Arisawa *et al.* 1990, Navarro *et al.* 2015). Peripheral (iv) administration of substance P inhibited tonic LH secretion in OVX rats (Vijayan and McCann 1979, Arisawa *et al.* 1990) and specific nucleus (intra-mPOA) administration of substance P inhibited tonic LH secretion in both intact and castrated male rats (Picanco-Diniz *et al.* 1990). Furthermore, icv administration of substance P had no effects on tonic LH release in intact female monkeys (Eckstein *et al.* 1980). Similarly, icv administration of substance P did not affect LH secretion in intact male rats or in OVX rats with or without E_2 replacement (Kalra *et al.* 1992, Sahu and Kalra 1992). The discrepancies among these studies might be due to the differences in the species, sex, sexual gonadal levels or methods (route and site) of drug delivery.

The studies described above showed either a net increase or decrease in mean levels of LH in response to substance P. However, they do not address the role of substance P in the regulation of pulsatile GnRH/LH release. Earlier studies have shown that neurones expressing *Tac1* mRNA (encoding substance P and NKA) were found in the ARC in rats (Larsen *et al.* 1993). Furthermore, along with its receptor (NK1R, encoded by gene *Tacr1*), substance P fibres were also shown to be concentrated in the ARC in rats (Shults *et al.* 1984, Buck *et al.* 1986, Hershey *et al.* 1991). Recent findings have underscored substance P as a

potential modulator of the GnRH pulse generator, since substance P is co-localised with kisspeptin and NKB in KNDy neurones in the human hypothalamus (Hrabovszky *et al.* 2013). However, in mice, *Tac1* mRNA measured by *in situ* hybridization was not co-expressed with *Kiss1* mRNA in the ARC (Navarro *et al.* 2015), indicating a possible species difference. Furthermore, substance P directly increases the excitability of mouse ARC Kiss1 neurones *in vitro* (de Croft *et al.* 2013) and the ARC Kiss1 neurones express *Tacr1* mRNA (Navarro *et al.* 2015). In addition, *Kiss1r* knockout mice reversed the stimulatory effect of substance P on LH release (Navarro *et al.* 2015). It is therefore reasonable to assume that Kiss1 signalling in the ARC modulates the effects of substance P on LH release.

5.2 Aims and objectives

To test the hypothesis that NK1R signalling in the ARC regulates the release of LH in female rats and this regulation is affected by the circulating levels of E_2.

a) To investigate the effects of intra-ARC administration of substance P or GR73632, a selective NK1R agonist, on LH release in OVX rats with (OVX+2×E_2; 36.8 ± 2.4 pg/ml, equal to circulating levels of oestrus phase of intact female rats) or without (OVX+0×E_2) E_2 replacement.

b) To determine whether activation of NK1R in the ARC by substance P or GR73632 modulates the frequency of pulsatile LH release in OVX rats without E_2 replacement.

5.3 Materials and methods

5.3.1 Animals and surgical procedures

Under general anaesthesia, adult Sprague-Dawley rats were OVX and implanted with bilateral guide cannulae directed towards the ARC (see section 2.2.2.5). In addition, animals receiving E_2 replacement were implanted subcutaneously with two Silastic^{TM} E_2 capsules (equal to circulating levels of oestrus phase of intact female rats; see Chapter 4), while animals without E_2 replacement were replaced with two Silastic^{TM} capsules filled with arachis oil (see section 2.2). After a 10-d recovery period (Fig. 5.1), rats were fitted with two indwelling cardiac catheters via the jugular vein, as described in section 2.2.2.6. Experimentation commenced 3 d later (Fig. 5.1). Cannula placement was verified by histological inspection at the end of the experiment, and animals with cannulae tips located outside the ARC were analysed separately.

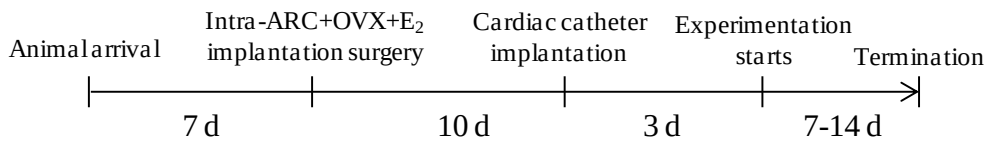

Fig. 5.1 Timeline diagram of various surgical and experimental procedures for investigating the effects of ARC substance P-NK1R signalling system on LH release in OVX rats replaced with different levels of E_2.

5.3.2 Experiment 1: Effects of substance P on LH release in OVX rats with or without E_2 replacement

On the morning of experimentation, a bilateral internal injection cannula (Plastics One) with extension tubing preloaded with substance P (Sigma-Aldrich) or vehicle (aCSF) was inserted into the guide cannula and extended 1 mm beyond the guide cannula to reach the ARC. The distal end of the tubing was extended outside of the cage connected to a 5 µl Hamilton syringe (Waters International), thereby allowing remote infusion without disturbing the rat during the experiment (see section 2.2.3.3). Rats were also attached via one of the two cardiac catheters to the computer-controlled automated blood sampling system (see section 2.2.3.1). Once connected, animals were left undisturbed for 1 h. Blood sampling commenced between 1000 and 1100 h and samples were collected every 5 min for 6 h for LH measurement.

Substance P (10 or 100 pmol in 400 nl aCSF per side, n = 7, 8, respectively) or aCSF (400 nl per side, n = 7) was administered bilaterally (intra-ARC) over 5

min after 2 h of controlled blood sampling into OVX+0×E_2 rats. In addition, a separate group of OVX+2×E_2 rats was injected (intra-ARC) with 400 nl aCSF (n = 5), 10 pmol substance P (n = 6), or 100 pmol substance P (n = 7). The dose of substance P was selected based on our previous study (Grachev *et al.* 2012a) showing that intra-ARC administration of senktide (1 or 10 pmol), a NK3R agonist, inhibited the pulsatile LH release. However, our preliminary study with 1 pmol of substance P failed to show any effects on LH release, which prompted us to adopt higher doses (10 and 100 pmol).

5.3.3 Experiment 2: Effects of GR73632, an agonist of NK1R, on LH release in OVX rats with or without E_2 replacement

On the morning of experimentation, the rats were attached to the automated blood sampling system and equipped with the intra-ARC injection system, as described above. After 2-h control blood sampling, GR73632 (10 or 100 pmol in 400 nl aCSF per side, n = 6, 7, respectively, Tocris Biosciences), a selective agonist of NK1R (Hagan *et al.* 1991), or aCSF (400 nl, n = 6) was administered (intra-ARC) over 5 min into OVX+0×E_2 rats. Additionally, GR73632 (10 or 100 pmol in 400 nl aCSF per side, n = 6, 8, respectively) or aCSF (400 nl per side, n = 5) was administered into a separate group of OVX+2×E_2 rats.

5.3.4 Statistical analysis

Detection of LH pulses in OVX+0×E_2 rats was established by use of the algorithm ULTRA (Van Cauter 1988). The effect of pharmacological agents on pulsatile LH secretion was analysed by comparing the mean LH pulse interval in the 2-h period preceding treatment with that in two consecutive 2-h post-treatment periods. The duration (min) of the 2-h pre-treatment, and the 1st and 2nd 2-h post-treatment periods, was divided by the number of LH pulses detected in each of these periods to give the appropriate LH pulse interval. The significance of the effect of treatments on LH pulse intervals was compared with control animals injected with vehicle, at the same time period, as well as with mean pulse interval during the 2-h pre-treatment period. The effect of pharmacological agents on overall LH release was calculated by comparing the AUC in the 1st and 2nd h post-treatment periods with that in the 1-h pre-treatment period, using SigmaPlot version 11 (Systat Software). Statistical significance was tested using one-way ANOVA followed by Dunnett's test. All data were shown as mean ± S.E.M. $P < 0.05$ was considered statistically significant.

5.4 Results

5.4.1 Experiment 1: Effects of substance P on LH release in OVX rats with or without E_2 replacement

To investigate the activation of NK1R on LH release in different E_2 levels, we

examined the effects of intra-ARC administration of substance P on LH secretion in OVX rats with or without E_2 replacement. Intra-ARC administration of substance P caused a dose-dependent inhibition of LH pulse interval in OVX+0×E_2 rats (Fig. 5.2, G). At the dose of 10 pmol, substance P prolonged the LH pulse interval by 44.2% (2-h pre-injection *vs.* 1st 2-h post-injection: 22.4 ± 1.0 *vs*. 32.3 ± 1.5 min; $P < 0.05$), and by 79.4% with 100 pmol substance P, (2-h pre-injection *vs.* 1st 2-h post-injection: 23.9 ± 0.7 *vs*. 42.9 ± 3.0 min; $P < 0.05$). LH pulse frequency recovered after suppression due to substance P, with pulse intervals returning to 23.0 ± 0.8 and 25.7 ± 2.1 min during the 2nd 2-h period after treatment with 10 pmol and 100 pmol substance P, respectively. In the control group, infusion of vehicle (aCSF) did not affect the LH pulse frequency.

Due to the negative feedback effect of E_2 with the replacement regime of 2 E_2 capsules, LH pulses were not evident. We therefore calculated the overall LH secretion by using AUC and, for comparative purposes, we used this method of analysis for both OVX+0×E_2 and OVX+2×E_2 rats treated with substance P. Intra-ARC administration of substance P (100 pmol) reduced AUC of the LH profile during the 2nd 1-h period after treatment in OVX+0×E_2 rats (1-h pre-injection *vs.* 2nd 1-h post-injection: 269.2 ± 6.8 *vs*. 224.4 ± 11.2 ng/ml.min; $P < 0.05$; Figs. 5.2, C and H). By contrast, AUC of the LH profile was increased during the 1st h post-administration period in OVX+2×E_2 rats (AUC in 1-h pre-injection *vs.* AUC in 1st 1-h post-injection: 155.4 ± 5.6 *vs*. 189.0 ± 3.3 ng/ml.min; $P < 0.05$; Figs. 5.2, F and H). However, this stimulation was

followed by an inhibition of LH secretion during the 2nd h post-administration period (AUC in 1-h pre-injection *vs*. AUC in 2nd 1-h post-injection: 155.4 ± 5.6 *vs*. 128.5 ± 7.1 ng/ml.min; $P < 0.05$; Figs. 5.2, F and H). Both substance P (10 pmol) and vehicle (aCSF) did not affect the LH secretion measured by AUC in OVX+2×E_2 rats, as well as in OVX+0×E_2 rats (Figs. 5.2, A, B, D, E and H). In addition, no misplaced cannulae were found in this set of experiments.

5.4.2 Experiment 2: Effects of GR73632, an agonist of NK1R, on LH release in OVX rats with or without E_2 replacement

To investigate whether substance P acts through NK1R to affect the LH secretion, we examined the effects of intra-ARC administration of GR73632, a selective NK1R agonist, on LH secretion in OVX rats with or without E_2 replacement. As was observed with substance P, intra-ARC administration of GR73632 caused a dose-dependent inhibition of pulsatile LH release in OVX+0×E_2 rats (Fig. 5.3, G). GR73632 prolonged the LH pulse interval by 49.6% with the 10 pmol dose (2-h pre-injection *vs.* 1st 2-h post-injection: 22.4 ± 1.9 *vs*. 33.5 ± 1.6 min; $P < 0.05$), and by 78.5% with the 100 pmol dose (2-h pre-injection *vs.* 1st 2-h post-injection, 22.3 ± 1.7 *vs*. 39.8 ± 3.5 min; $P < 0.05$). After treatment with 10 pmol or 100 pmol of GR73632, LH pulse frequency recovered during the 2nd 2-h post-treatment period, with pulse intervals returning to 21.9 ± 1.2 and 24.9 ± 0.9 min, respectively. In the control group, infusion of vehicle (aCSF) did not affect

the LH pulse frequency.

Due to the negative feedback effect of E_2 with the replacement regime of 2 E_2 capsules, LH pulses were not evident. We therefore calculated the overall LH secretion by using AUC and, for comparative purposes, we used this method of analysis for both OVX+0×E_2 and OVX+2×E_2 rats treated with GR73632. Intra-ARC administration of GR73632 (100 pmol) reduced the AUC of the LH profile in OVX+0×E_2 rats (1-h pre-injection *vs*. 2nd 1-h post-injection: 269.0 ± 6.9 *vs*. 219.4 ± 11.7 ng/ml.min; $P < 0.05$; Figs. 5.3, C and H). By contrast, LH levels were increased during the 1st h post-administration period in OVX+2×E_2 rats (AUC in 1-h pre-injection *vs*. AUC in 1st 1-h post-injection: 153.8 ± 3.4 *vs*. 182.4 ± 9.8 ng/ml.min; $P < 0.05$; Figs. 5.3, F and H). This simulation was followed by an inhibition during the 2nd h post-administration period (AUC in 1-h pre-injection *vs*. AUC in 2nd 1-h post-injection: 153.8 ± 3.4 *vs*. 118.0 ± 8.1 ng/ml.min; $P < 0.05$; Figs. 5.3, F and H). Both GR73632 (10 pmol) and vehicle (aCSF) did not affect the LH secretion measured by AUC in OVX+2×E_2 rats, as well as in OVX+0×E_2 rats (Figs. 5.3, A, B, D, E and H). ARC cannulae were found to be misplaced in two OVX+2×E_2 rats (Fig. 5.4). GR73632 did not affect the LH secretion in those two rats at the dose of 10 pmol (data not shown) or 100 pmol (AUC in 1-h pre-injection *vs*. AUC in 1st 1-h post-injection *vs*. AUC in 2nd 1-h post-injection: 156.8 ± 2.1 *vs*. 161.4 ± 4.3 *vs*. 150.6 ± 2.5 ng/ml.min, $P > 0.05$).

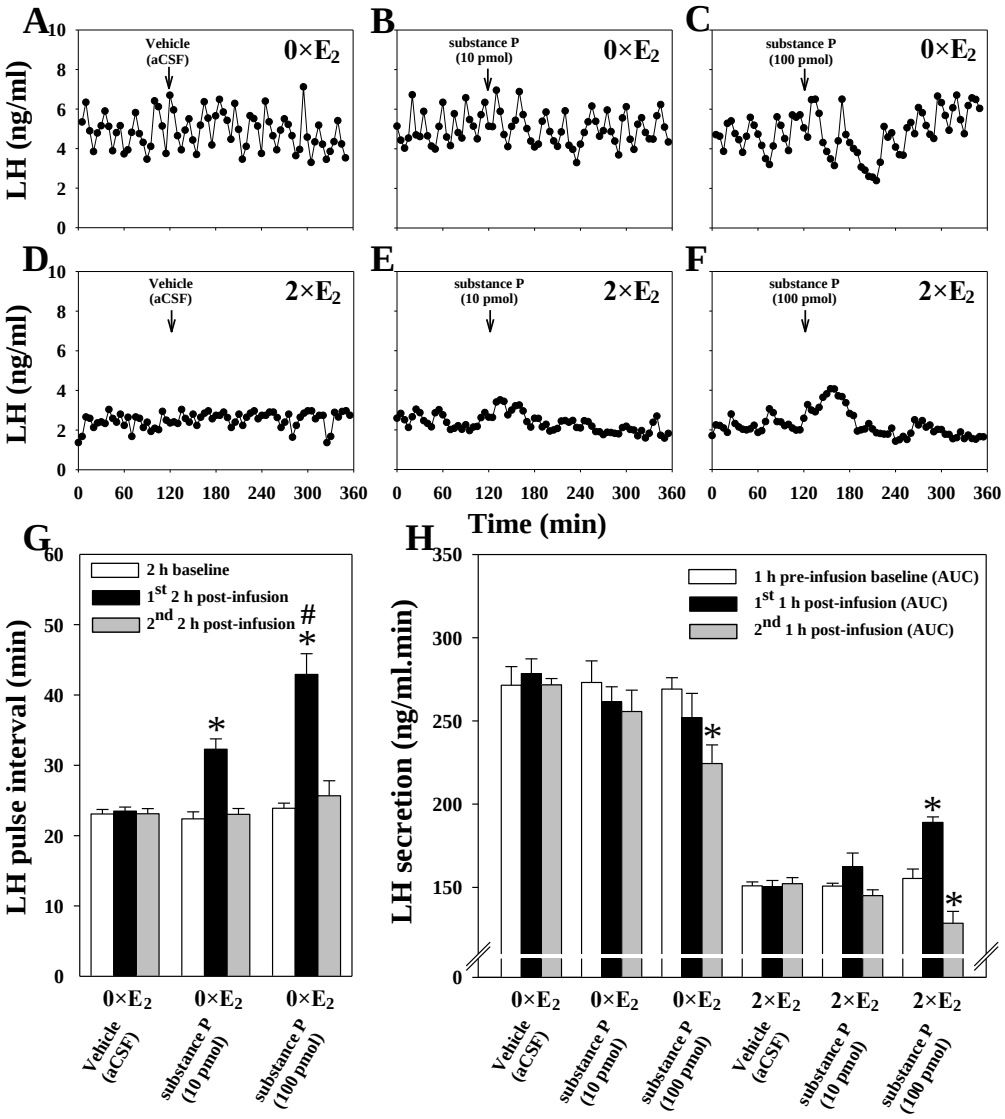

Fig. 5.2 Effects of substance P on LH release in OVX rats with or without E_2 replacement. Representative LH profiles demonstrating (1) effects of intra-ARC administration (arrow) of substance P or vehicle (aCSF) on pulsatile LH secretion in OVX rats without E_2 replacement (labelled as $0\times E_2$) (A-C), and (2) effects of intra-ARC administration of substance P or vehicle (aCSF) on LH secretion in OVX rats replaced with two E_2 capsules (labelled as $2\times E_2$) (D-F). Central administration of substance P dose-dependently suppressed pulsatile LH release in OVX+$0\times E_2$ rats, as summarised in panel G. 100 pmol substance P inhibited LH secretion (calculated by AUC) in OVX+$0\times E_2$ rats (H). Furthermore, 100 pmol substance P stimulated LH secretion in OVX+$2\times E_2$ rats (H). However, this stimulation was followed by an inhibition (H). The effects of substance P on LH secretion are summarised in panel H. *$P < 0.05$ *vs.* 1-h baseline within the same group, as well as the same 1-h period within the vehicle treated group; $^{\#}P < 0.05$ *vs.* same 1-h period within the group treated with 10 pmol substance P (n = 5-8 per group).

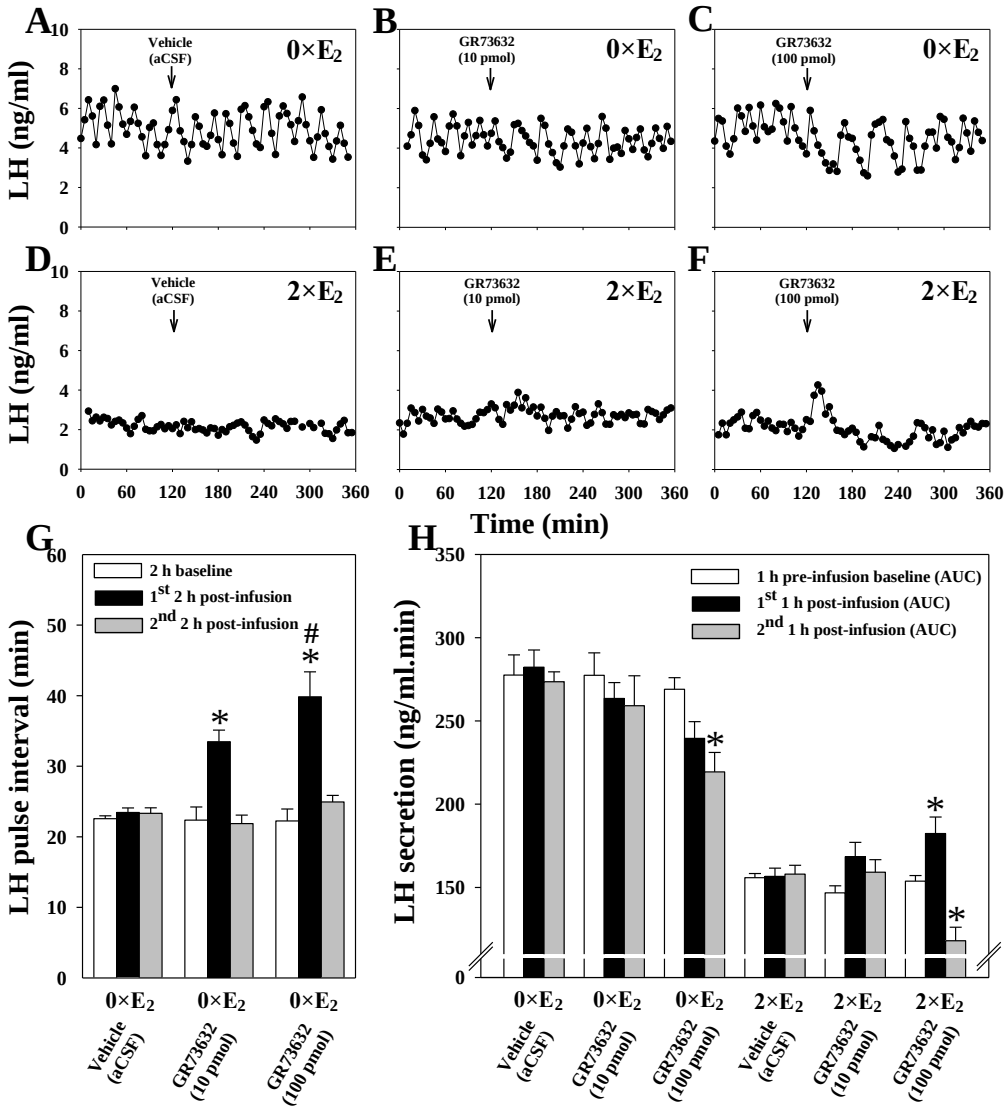

Fig. 5.3 Effects of GR73632, an agonist of NK1R, on LH release in OVX rats with or without E_2 replacement. Representative LH profiles demonstrating (1) effects of intra-ARC administration (arrow) of GR73632 or vehicle (aCSF) on pulsatile LH secretion in OVX rats without E_2 replacement (labelled as $0\times E_2$) (A-C), and (2) effects of intra-ARC administration of substance P or vehicle (aCSF) on LH secretion in OVX rats replaced with two E_2 capsules (labelled as $2\times E_2$) (D-F). Central administration of GR73632 dose-dependently suppressed the pulsatile LH release in OVX+$0\times E_2$ rats, as summarised in panel G. 100 pmol GR73632 inhibited LH secretion (calculated by AUC) in OVX+$0\times E_2$ rats (H). Furthermore, 100 pmol GR73632 stimulated LH secretion in OVX+$2\times E_2$ rats (H). However, this stimulation was followed by an inhibition (H). The effects of GR73632 on LH secretion are summarised in panel H. *$P < 0.05$ *vs.* 1-h baseline within the same group, as well as the same 1-h period within the vehicle treated group; $^{\#}P < 0.05$ *vs.* same 1-h period within the group treated with 10 pmol GR73632 (n = 5-8 per group).

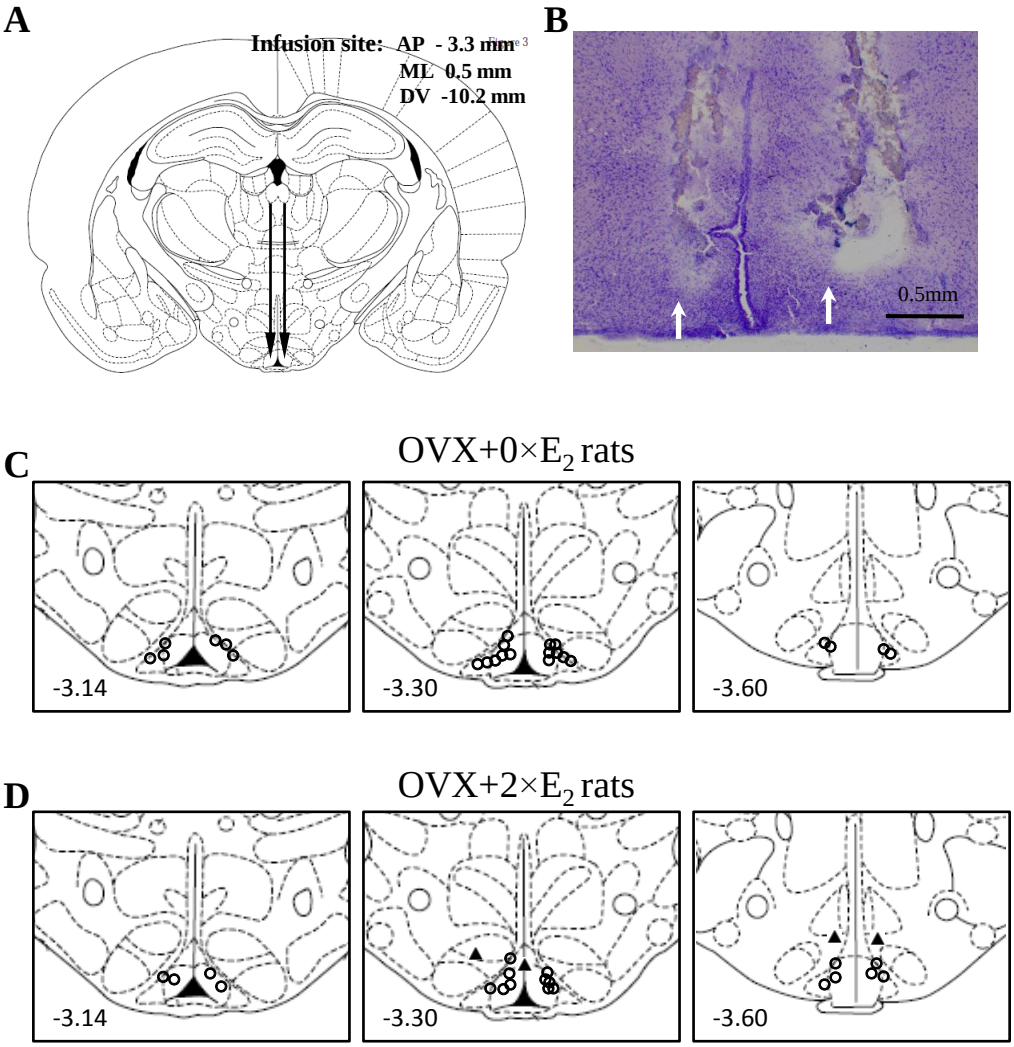

Fig. 5.4 Schematic illustration and photomicrograph of the infusion site targeted to the ARC. A, schematic illustration showing the target site for bilateral cannulation of the ARC. Arrows point to the location of the tips of internal cannulae. B, photomicrograph of a coronal brain section in a representative animal implanted with a bilateral cannula in the ARC. Arrows indicate the tip of the internal cannulae. C-D, schematic drawings of the ARC illustrating the individual sites of injection for the experiments carried out in OVX+0×E_2 and OVX+2×E_2 rats, respectively. Numbers in each drawing indicate the distance (mm) to Bregma. Open circles show the right injection sites. Close triangles show the misplaced injection sites.

5.5 Discussion

The present study has shown that activation of NK1R in the ARC stimulated LH release in conditions of high circulating levels of E_2 (equivalent to those observed in the oestrus phase of the cycle). In contrast, an inhibitory response was observed in conditions of low E_2 levels (OVX without E_2 replacement). These results support previous studies showing a similar biphasic effects of iv administration of substance P on LH release (Arisawa *et al.* 1990) and therefore indicate the ARC to be the primary site of action. Although substance P is not indicated to cross the blood-brain barrier, it is possible that iv administration may still act at the level of the ARC, since this structure, which lies immediately dorsal to the ME, is one of the circumventricular organs characterised by an incomplete blood-brain barrier and therefore susceptible to the peripheral hormone environment (Yamamura *et al.* 2015). If the above hypothesis is correct, then the inhibitory effects of iv administered substance P observed in previous studies in OVX rats without E_2 replacement (Vijayan and McCann 1979, Arisawa *et al.* 1990) could be explained by the activation of NK1R in the ARC, as shown in the present study. However, these biphasic effects were not demonstrated in a recent study which showed that the activation of NK1R (icv administration) stimulated LH release in OVX mice with or without E_2 replacement (Navarro *et al.* 2015). These conflicting data may be due to the species differences or different routes of drug delivery. Nevertheless, the results from an earlier study showed that substance P induced LH release follow icv

administration, but inhibited LH release following iv administration in OVX rats without E_2 replacement (Vijayan and McCann 1979).

The results of the present study are similar to those examining the role of NK3R activation in the regulation of LH release, as described in Chapter 4. Similar to NKB, the underlying mechanism for the stimulatory effects of substance P on the LH release implicate Kiss1 signalling in the ARC. Recent studies showed that substance P fibres innervated Kiss1 neurones in the ARC in sheep and monkeys (Bruna *et al.* 2014, Chrysanthi *et al.* 2015). Furthermore, *Kiss1r* knockout mice were devoid of substance P-induced LH release and ARC Kiss1 neurones express NK1R in mice (Navarro *et al.* 2015). Since intra-ARC administration of the NK1R agonists induced LH secretion, as shown in the present study, it is reasonable to postulate that substance P may stimulate LH release via KNDy neurones in the ARC.

The present study has also shown that this transitory stimulatory effect was followed by an inhibition of LH release. This inhibitory effect was not observed with NK3R and NMDA agonists (see Chapter 4), although a tendency towards inhibition was evident. The specific location of administration of NK1R agonists into the ARC may account for the more obvious inhibitory effect, although the underlying mechanisms and physiological relevance for this kind of inhibition remain uncovered.

We have also shown that intra-ARC administration of the NK1R agonist dose-dependently suppressed pulsatile LH release in OVX+0×E_2 rats. This indicates that substance P may inhibit the GnRH pulse generator in conditions of low E_2. Morphological studies have demonstrated that substance P may innervate KNDy neurones in the ARC which are postulated to be an essential component of the GnRH pulse generator (Lehman *et al.* 2010). It is therefore possible that substance P inhibits the pulsatile LH release via the KNDy neurones. Furthermore, previous studies have shown that activation of NK3R in the ARC suppressed pulsatile LH release (Grachev *et al.* 2012a, Grachev *et al.* 2014a). The NK3R agonist-induced suppression of LH pulses is thought to be mediated via Dyn signalling in the ARC (Grachev *et al.* 2012a). It is possible that the inhibitory effect of NK1R agonism on LH pulses is also mediated through Dyn signalling.

In order to exclude the possibility that substance P act on receptors other than NK1R to affect LH release, we used the selective NK1R agonist GR73632 (Navarro *et al.* 2015, Ruiz-Pino *et al.* 2015, Simavli *et al.* 2015). Previous studies have shown GR73632 may be more potent on NK1R compared to substance P (Hagan *et al.* 1991). However, the effects of substance P and GR73632 on the dynamics of LH secretion in the present study were very similar. This might suggest that substance P may have a stronger affinity with NK1R compared with NK2R or NK3R (Seabrook *et al.* 1995). Compared with NK3R agonists (Grachev *et al.* 2012a), the effects of intra-ARC administration of the

NK1R agonists (even with a 10 times higher dose) on LH release in the ARC were very modest. This could be due to a more robust expression of NK3R in the KNDy neurones compared with NK1R (Navarro *et al.* 2015). It is possible that NK1R signalling may play a less important role in regulating the GnRH pulse generator compared with NK3R. This is supported by a recent study showing that a 100 times higher dose of NK1R agonists (compared to NK3R agonists) was required to induce the MUA volley (an electrophysiological correlate of GnRH pulse generator activities) in OVX goats (Yamamura *et al.* 2015). Although the morphological and pharmacological studies supported the potential role of NK1R signalling in the regulation of the GnRH pulse generator, the physiological significance of substance P in the reproductive system warrants cautious interpretation. Nevertheless, a recent study showed that *Tac1* knockout mice presented a delayed puberty onset without changing the body weight (Simavli *et al.* 2015). An acceleration of the GnRH pulse generator is required for the initiation of puberty (Sisk and Foster 2004). It could be inferred that substance P may be involved in the acceleration of the GnRH pulse generator during the prepubertal stage, which is in keeping with the concurrent increased substance P signalling during this period (Simavli *et al.* 2015).

In summary, the present study has shown biphasic effects of substance P on LH release, depending on the prevailing levels of E_2. Furthermore, we determined the ARC to be a primary site of action, which suggests that the KNDy neural system may be a downstream effector of substance P signalling.

CHAPTER SIX: RFRP-3 MODULATES BASAL AND STRESS-INDUCED SUPPRESSION OF PULSATILE LH RELEASE IN FEMALE RATS

6.1 Introduction

GnIH was discovered in birds to be the first hypophysiotrophic hormone to directly inhibit the gonadotrophin secretion at the level of the anterior pituitary gland (Tsutsui *et al.* 2000). Its mammal orthologue, RFRP, was later identified and characterised in a variety of mammal species, including rodents (Ukena *et al.* 2002, Kriegsfeld *et al.* 2006). However, unlike their avian orthologue, the hypophysiotrophic role of RFRP on gonadotrophin release in rodents has been extensively questioned since RFRP fibres are sparse or absent in external layer of ME (Kriegsfeld *et al.* 2006, Johnson *et al.* 2007, Rizwan *et al.* 2009), and RFRP neurones are unable to absorb retrograde tract tracing dye injected peripherally (Rizwan *et al.* 2009). Therefore, it is unlikely that RFRP is released into the portal veins to act on anterior pituitary. Instead, RFRP is indicated as a hypothalamic neuropeptide since mRNA of both *RFRP* and *GPR147* (RFRP receptor) were predominantly expressed in hypothalamus in rodents compared with other tissues such as pituitary gland and gonads (Hinuma *et al.* 2000).

The *RFRP* mRNA encodes a polypeptide that is cleaved into two neuropeptides (RFRP-1 and RFRP-3) (Hinuma *et al.* 2000, Ukena *et al.* 2002, Tsutsui *et al.* 2012). Whilst RFRP-1 does not exert an inhibitory effect on the release of

gonadotrophins in rodents (Pineda *et al.* 2010b), numerous studies have demonstrated that central administration of RFRP-3 is able to exert an inhibitory effect on the HPG axis, principally a robust reduction in mean circulating levels of LH (Kriegsfeld *et al.* 2006, Johnson *et al.* 2007, Kirby *et al.* 2009, Pineda *et al.* 2010b, Leon *et al.* 2014), which suggests RFRP-3 is likely to be the GnIH in mammals, but with a different site of action. Furthermore, RFRP-3-induced LH suppression may act through the GPR147 receptor, since this inhibitory effect was nullified in *GPR147* knockout mice (Leon *et al.* 2014). However, other studies investigating the mechanisms underlying this inhibition have yielded absence of effect of RFRP-3 on pulsatile LH secretion, including LH pulse frequency and amplitude (Murakami *et al.* 2008, Anderson *et al.* 2009). These above results are at odds with the inhibitory role of RFRP-3 on LH release and therefore warranted further investigation. A recent *in vitro* study used fast-scan cyclic voltammetry to directly detect the oxidation of spontaneously secreted GnRH at the level of ME in prepubertal male mice (Glanowska *et al.* 2014). It showed that RFRP-3 reduced frequency and amplitude of GnRH pulses (Glanowska *et al.* 2014). Furthermore, 1-adamantanecarbonyl-Arg-Phe-NH_2 trifluoroacetate (RF-9), a RFRP-3 antagonist (Simonin *et al.* 2006, Pineda *et al.* 2010c), induced a significant increase in the frequency of GnRH pulses, but not the amplitude (Glanowska *et al.* 2014). It was therefore suggested that RFRP-3 may function as a brake on the GnRH pulse generator in prepubertal animals and this modulation may be regulated specifically through GnRH pulse frequency

and/or amplitude. However, no *in vivo* study has yet established the role of hypothalamic RFRP-3 signalling in the regulation of GnRH pulse generator activity in adult mammals.

It is well known that various stressful stimuli profoundly inhibit the HPG axis through suppression of pulsatile GnRH release and therefore pulsatile LH release (Ferin 1993), although the underlying mechanisms are not fully elucidated. CRF, an important stress modulator, has been implicated in this process since CRF antagonist blocks the stress-induced suppression of pulsatile LH release (Rivier *et al.* 1986, Cates *et al.* 2004, Li *et al.* 2006). Although central administration of CRF decreases the release of GnRH into hypophysial portal veins (Petraglia *et al.* 1987), CRF neurones do not appear to innervate GnRH neurones directly, since *CRF-R1* (encoding type 1 CRF receptor) mRNA do not co-express with *GnRH* mRNA in rats (Hahn *et al.* 2003). CRF neurones located in major CRF expressing nuclei fail to pick up the retrograde tracer injected into the vicinity of the GnRH neurones (Hahn *et al.* 2003). Alternatively, CRF may innervate KNDy neurones in the ARC to modulate the GnRH pulse generator. Both CRF-R1 and R2 are expressed in the ARC (Chalmers *et al.* 1995, Van Pett *et al.* 2000) and central administration of CRF decreases *Kiss1* and *Kiss1r* mRNA expression in the ARC (Kinsey-Jones *et al.* 2009). However, it is unknown whether CRF mediate the Kiss1 signalling in the ARC directly, since the expression of CRF receptors has not been found in KNDy neurones. It is therefore possible that CRF-mediated suppression of the GnRH signalling and/or

GnRH pulse generator may involve indirect regulatory mechanisms. The inhibitory effects of RFRP-3 on the HPG axis raise the question of whether stress-induced suppression of LH release operates through this system. Indeed, the identification of CRF neurones and fibres in the DMH (Cummings *et al.* 1983) and co-expression of CRF-R1 on RFRP neurones (Kirby *et al.* 2009) using immunohistochemical labelling provides an anatomical structure for the interaction between CRF and RFRP signalling systems. Furthermore, CRF signalling has been implicated in regulating different types of stressors-induced LH suppression in rats since CRF-R1 antagonism blocks restraint-induced LH suppression and CRF-R2 antagonism blocks both restraint and LPS-induced LH suppression (Li *et al.* 2006). It is therefore reasonable to speculate that RFRP signalling may also play a role in psychological and immunological stressors-induced LH suppression. Accordingly, it has been shown that both immobilisation and LPS stress increases *RFRP* mRNA expression in the DMH, which is associated with inhibition of LH secretion in rats (Kirby *et al.* 2009, Iwasa *et al.* 2014).

RFRP-3 may act as a possible downstream of CRF signalling and a possible mediator of the basal and/or stress-induced suppression of the GnRH pulse generator. It is reminiscent of another important hypothalamic neuropeptide signalling: the endogenous opioid peptides. Endogenous opioid peptides have been implicated in the suppression of pulsatile LH release with opioid receptor antagonists increasing and opioid receptor agonist suppressing the frequency of

the GnRH pulse generator in rats (Kimura *et al.* 1991, Grachev *et al.* 2012a, Nakahara *et al.* 2013). Furthermore, opioid receptor antagonists blocked the stress-induced suppression of pulsatile LH release in both monkeys and rats (O'Byrne *et al.* 1989, Cagampang and Maeda 1991). Accordingly, CRF-induced suppression of LH release can also be mediated via endogenous opioid peptides and this mediation may involve the μ-opioid receptor (Gindoff and Ferin 1987, Almeida *et al.* 1988, Rivest *et al.* 1993). However, it remains unknown whether the endogenous opioid peptides mediate the inhibitory effects of RFRP-3 on the pulsatile LH release.

RFRP-3 exerts an inhibitory effect on the HPG axis, which prompted us to investigate whether this effect is mediated through specific locations in the brain. Morphological evidences have shown that RFRP-3 neurones project into the ARC to innervate 35% of Kiss1 neurones and approximate 25% of KNDy neurones in the ARC co-express *GPR147* mRNA (Poling *et al.* 2013). Furthermore, RFRP-3 has an inhibitory effect on Kiss1 neurones in the ARC, since the number of *Kiss1* mRNA expressing neurones in the ARC increased by 60% in *GPR147* knockout mice (Leon *et al.* 2014). Due to the fact that KNDy neurones in the ARC have been implicated as the putative GnRH pulse generator, it is possible that RFRP-3 inhibit the KNDy neurones in the ARC to suppress the pulsatile LH release.

Alternatively, another major site of action of RFRP-3 at the HPG axis can be the mPOA, where most GnRH neurones are located. A number of studies have

shown that RFRP-3 fibres form close apposition with 25 to 75% of GnRH neurones (Kriegsfeld *et al.* 2006, Johnson *et al.* 2007, Rizwan *et al.* 2012, Ubuka *et al.* 2012). Furthermore, 33% of GnRH neurones in the mPOA co-express *GPR147* mRNA (Rizwan *et al.* 2012). RFRP-3-induced LH suppression via GPR147 receptor is dependent on the GnRH signaling, since the stimulatory effect of RF-9 is blocked by pre-treatment of a GnRH receptor antagonist (Rizwan *et al.* 2012). Furthermore, *in vitro* electrophysiological studies have shown that RFRP-3 directly suppresses the excitability of GnRH neurones and blocks the stimulatory action of Kiss1 on GnRH neurones (Ducret *et al.* 2009, Wu *et al.* 2009). It is therefore evident that mPOA could play an essential role in RFRP-3-induced inhibition of LH release through a direct interaction, even though this process may not directly involve the GnRH pulse generator located in the ARC.

RFRP have also been implicated in negative feedback control of the reproductive axis in females (Kriegsfeld *et al.* 2006, Leon *et al.* 2014). Previous studies have shown that 25 to 40% of RFRP neurones co-express ER-α receptors and peripheral treatment of E_2 induce a robust c-Fos expression in RFRP neurones (Kriegsfeld *et al.* 2006, Poling *et al.* 2012). This suggests a direct action of E_2 on activities of RFRP neurones. ERα receptors are involved in E_2 negative feedback control of LH secretion and *GnRH* mRNA expression in mice (Dorling *et al.* 2003). Therefore, E_2-induced LH suppression may be mediated via RFRP-3 signalling. Indeed, recent studies have shown that the removal of

ovaries induced a higher LH level in *GPR147* knockout mice compared with wild type mice (Leon *et al.* 2014). It suggests GPR147 plays an inhibitory role in LH release when E_2 levels are normal. Even though there is a considerable amount of evidence for the involvement of RFRP-3 in the E_2 negative feedback control, few studies have explored the underlying mechanisms. Given its direct innervations of both GnRH and KNDy neurones, RFRP-3 may regulate E_2-induced negative feedback control of GnRH release in the same mode, with direct action on GnRH neurones and/or indirect regulation through KNDy neurones, which have also been implicated in the E_2-induced negative feedback control of the HPG axis (d'Anglemont de Tassigny and Colledge 2010, Hameed *et al.* 2011).

6.2 Aims and objectives

To test the hypothesis that RFRP-3 signalling regulates both basal and stress-induced suppression of pulsatile LH release and mediates the E_2-induced negative feedback control of LH release in female rats.

1. To determine the role of RFRP-3 signalling in the control of the GnRH pulse generator in OVX rats.

a) To identify the effect of central administered RFRP-3, as well as its antagonist RF-9, on the pulsatile release of LH.

b) To investigate whether pre-treatment of RF-9 blocks the effect of RFRP-3 on LH pulses.

c) To assess whether the effect of central administered RFRP-3 on pulsatile LH secretion is modulated through endogenous opioid peptide signalling, and to determine the opioid receptor subtypes involved.

2. To elucidate the role of RFRP-3 signalling in stress-induced suppression of the GnRH pulse generator in OVX rats.

a) To determine whether central administration of RF-9 blocks CRF-induced suppression of pulsatile LH release.

b) To determine whether central administration of RF-9 blocks restraint and LPS stress-induced suppression of LH pulses.

3. To evaluate the mechanisms and sites of action of RFRP-3 in the negative feedback control of E_2 on LH secretion.

a) To test whether the effect of RFRP-3 on pulsatile LH release is mediated through the ARC and to determine whether the RFRP-3 signalling in the ARC is involved in the E_2-induced suppression of LH release by administering RRFP-3 and RF-9 into the ARC of both OVX rats with (2 E_2

capsules; equal to oestrus levels of E_2; see Chapter 4) or without E_2 replacement.

b) To investigate whether the inhibitory effect of RFRP-3 on LH release is mediated through the mPOA and to determine whether the RFRP-3 signalling in the mPOA is involved in the E_2-induced suppression of LH by administering RRFP-3, as well as RF-9, into the mPOA of both E_2 replaced and non-E_2 replaced OVX rats.

6.3 Materials and methods

6.3.1 Animals and surgical procedures

Adult Sprague-Dawley rats were implanted with unilateral icv guide cannulae directed towards left lateral cerebral ventricles (see section 2.2.2.4). Rats were also OVX without implanting any capsules. After a 10-d recovery period (Fig. 6.1), rats were all fitted with two indwelling cardiac catheters via the jugular vein, as described in section 2.2.2.6. Experimentation commenced 3 d later (Fig. 6.1).

A separate group of adult Sprague-Dawley rats were OVX and implanted with bilateral guide cannulae directed towards ARC or mPOA (see section 2.2.2.5). In addition, animals receiving E_2 replacement were implanted subcutaneously with two Silastic^{TM} E_2 capsules (equal to oestrus levels of E_2; see Chapter 4),

identified in the present study as 'OVX+2×E_2' rats. Animals without E_2 replacement were implanted with two Silastic^{TM} capsules filled with arachis oil and identified as 'OVX+0×E_2' rats. Rats were further fitted with two indwelling cardiac catheters via the jugular vein after a 10-d recovery period (Fig. 6.1). Experimentation commenced 3 d later (Fig. 6.1).

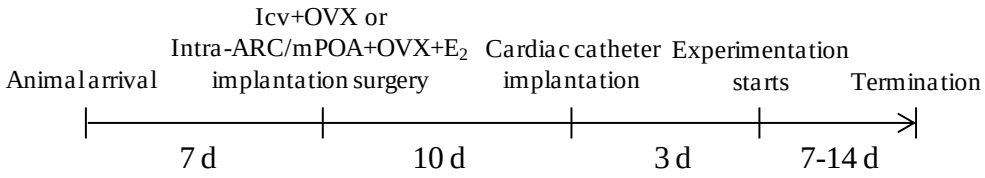

Fig. 6.1 Timeline diagram of various surgical and experimental procedures for investigating the role of RFRP-3 signalling in regulating the pulsatile LH release and the E_2-induced negative feedback control of LH release in female rats.

6.3.2 Experiment 1: Effects of RFRP-3 on pulsatile LH release in OVX rats

An icv injection cannula (Plastics One) with extension tubing preloaded with RFRP-3 (Bachem, Germany) was inserted into the guide cannula, with 1 µl of air separating the fluid in the tubing from the lateral ventricle system before the actual injection. The tip of injection cannula extended 1 mm beyond the guide cannula to reach the ventricle. The distal end of the tubing was extended outside of the cage connected to a 25 µl Hamilton syringe (Waters International), thereby allowing remote infusion without disturbing the rat during the

experiment (see section 2.2.3.3.1). Rats were also attached via one of the two cardiac catheters to a computer-controlled automated blood sampling system, which allows for the intermittent withdrawal of small blood samples (see section 2.2.3.1). Once connected, animals were left undisturbed for 1 h before sampling commenced between 1000 and 1100 h, and samples were collected every 5 min for 6 h.

To examine the effects of RFRP-3 on pulsatile LH release, RFRP-3 or vehicle (aCSF) was infused (icv) over 5 min after 2 h of controlled blood sampling into OVX rats. Rats received a single dose of 0.1, 1, or 5 nmol RFRP-3 in 4 µl aCSF in each treatment group (n = 5-8 per group) or 4 µl aCSF in the control group (n = 6). The doses of RFRP-3 were selected from a previous study (Pineda *et al.* 2010b).

6.3.3 Experiment 2: Effects of RF-9 on LH release in OVX rats

On the morning of experimentation, OVX rats were attached to the automated blood sampling system and equipped with the icv injection system, as described above. After 2 h of controlled blood sampling, rats were administered (icv) a single dose of 0.1, 1, or 10 nmol RF-9 (Sigma-Aldrich) in 4 µl aCSF in each treatment group (n = 5-7 per group) or 4 µl aCSF in the control group (n = 5). The doses selections were adopted from a previous study (Pineda *et al.* 2010c).

6.3.4 Experiment 3: Effects of RFRP-3 on pulsatile LH release in the presence of RF-9 in OVX rats

On the morning of experimentation, OVX rats were attached to the automated blood sampling system and equipped with the icv injection system, as described above. After 1 h 45 min of controlled blood sampling, rats received three consecutive injections (icv) of RF-9 (10 nmol in 4 µl aCSF, n = 8) or vehicle (4 µl aCSF, n = 8), each administered over 5 min, 30 min apart, with a further injection (icv) of RFRP-3 (5 nmol in 4 µl aCSF) administered 15 min following the first injection of RF-9 or aCSF. Additional rats, used as controls, received three consecutive injections (icv, 30 min apart) of RF-9 (10 nmol, n = 6) or aCSF (4 µl, n = 6) after 1 h 45 min of controlled blood sampling.

6.3.5 Experiment 4: Effects of RF-9 on CRF or stress-induced inhibition of pulsatile LH release in OVX rats

On the morning of experimentation, OVX rats were attached to the automated blood sampling system and equipped with the icv injection system, as described above. After 1 h 45 min of control blood sampling, rats received three consecutive injections (icv) of RF-9 (10 nmol in 4 µl aCSF, n = 8) or vehicle (4 µl aCSF, n = 6), each administered over 5 min, 30 min apart, with a further injection (icv) of CRF (2 nmol in 4 µl aCSF, Sigma-Aldrich), administered 15 min following the first injection of RF-9 or aCSF. Additional rats, used as

controls, received four consecutive injections (icv) of 4 µl aCSF (at 1 h 45 min, 2 h, 2 h 15 min and 2 h 45 min of blood sampling, n = 5).

Separate groups of OVX rats prepared as above were used for stress studies. For restraint stress studies, rats received three consecutive injections (icv) of RF-9 (10 nmol in 4 µl aCSF, n = 8) or vehicle (4 µl aCSF, n = 7) after 1 h 45 min of controlled blood sampling, each administered over 5 min, 30 min apart. Fifteen min after the first injection of RF-9 or aCSF, rats were placed in a restraint device for 1 h and blood sampling continued uninterrupted during the 1-h restraint and the 3-h post-restraint period (see section 2.2.3.2). Control animals (n = 5) received three consecutive injections (icv) of aCSF (4 µl) without being restrained. For LPS stress studies, rats received three consecutive injections (icv) of RF-9 (10 nmol in 4 µl aCSF, n = 9) or vehicle (4 µl aCSF, n = 8) after 1 h 45 min of controlled blood sampling. Fifteen min after the first injection of RF-9 or aCSF, rats were infused (iv) of LPS (15 µg/kg in 0.2 ml saline, Sigma-Aldrich) over 5 min via one of the two cardiac catheters. Additional control rats (n = 5) were infused (iv) of saline (0.2 ml) with three injections (icv) of aCSF (4 µl).

6.3.6 Experiment 5: Effects of opioid receptor antagonists on RFRP-3-induced inhibition of pulsatile LH release in OVX rats

On the morning of experimentation, OVX rats were attached to the automated blood sampling system and equipped with the icv injection system, as described

above. After 1 h 45 min of controlled blood sampling, rats were administered (icv) over 5 min with naloxone (30 nmol in 4 µl aCSF, a specific µ-receptor antagonist at this dose, n = 11), nor-BNI (6.8 nmol in 4 µl aCSF, a specific κ-receptor antagonist, n = 11) or naltrindole (12 nmol in 4 µl aCSF, a specific δ-receptor antagonist, n = 8). Fifteen min later, rats were further administered (icv) with RFRP-3 (5 nmol in 4 µl aCSF). The doses for the endogenous opioid receptor antagonists were from one of our previous studies (Bowe *et al.* 2005). Additional control rats were administered (icv) with vehicle (4 µl aCSF, n = 6), naloxone (30 nmol, n = 7), nor-BNI (6.8 nmol, n = 6), or naltrindole (12 nmol, n = 6) after 1 h 45 min control blood sampling, followed by vehicle (4 µl aCSF) 15 min later.

6.3.7 Experiment 6: Effects of intra-ARC administration of RFRP-3 or RF-9 on LH release in OVX rats with or without E_2 replacement

On the morning of experimentation, a bilateral internal injection cannula (Plastics One) with extension tubing preloaded with vehicle, RFRP-3 or RF-9 was inserted into the guide cannula and extended 1 mm beyond the guide cannula to reach the ARC. The distal end of the tubing was extended outside of the cage connected to a 5 µl Hamilton syringe (Waters International), thereby allowing remote infusion without disturbing the rat during the experiment (see

section 2.2.3.3.2). Rats were also attached to the automated blood sampling system, as described above.

Rats were administered bilaterally (intra-ARC) with vehicle (400 nl aCSF, n = 4), RFRP-3 (500 pmol in 400 nl aCSF, n = 6) or RF-9 (1 nmol in 400 nl aCSF, n = 8) over 5 min after 2 h of controlled blood sampling in OVX+0×E_2 rats. The doses used here is 1/10 of the maximal single doses used in the icv studies. In addition, a separate group of OVX+2×E_2 rats were injected bilaterally (intra-ARC) with 400 nl aCSF (n = 4), 500 pmol RFRP-3 (n = 6) or 1 nmol RF-9 (n = 6).

6.3.8 Experiment 7: Effects of intra-mPOA administration of RFRP-3 or RF-9 on LH release in OVX rats with or without E_2 replacement

The rats were similarly equipped with the intra-mPOA injection system and attached to the automated blood sampling system, as described above. After 2 h of controlled blood sampling, vehicle (400 nl aCSF, n = 4), RFRP-3 (500 pmol in 400 nl aCSF, n = 6) or RF-9 (1 nmol in 400 nl aCSF, n = 7) were administered bilaterally over 5 min (intra-mPOA) into OVX+0×E_2 rats. Additionally, vehicle (400 nl aCSF, n = 4), RFRP-3 (500 pmol in 400 nl aCSF, n = 6) or RF-9 (1 nmol in 400 nl aCSF, n = 6) were administered bilaterally (intra-mPOA) into a separate group of OVX+2×E_2 rats.

6.3.9 Statistical analysis

Detection of LH pulses in OVX rats in experiments 1-5 and OVX+0×E_2 rats in experiments 6-7 was established by use of the algorithm ULTRA (Van Cauter 1988). The effects of pharmacological agents on pulsatile LH secretion were analysed by comparing the mean LH pulse interval in the 2-h period preceding treatment with that in two consecutive 2-h post-treatment periods. The duration (min) of the 2-h pre-treatment, and the 1st and 2nd 2-h post-treatment periods, was divided by the number of LH pulses detected in each of these periods to give the appropriate LH pulse interval. The effect of pharmacological agents on LH pulse amplitude was also calculated in the same time periods used for LH pulse frequency. The LH amplitude was calculated from the differences between the nadir and apex of each LH pulses. The significance of the effects of treatments on LH pulse intervals and amplitude were compared with control animals injected with vehicle, at the same time period, as well as with mean pulse interval during the 2-h pre-treatment period. The effect of stress (restraint or LPS)-induced inhibition of pulsatile LH secretion was calculated by comparing the 1st prolonged pulse interval after stress with the mean pulse interval during the 2-h pre-stress period, as well as with control animals injected with vehicle at the same time points. The post-stress recovery period was defined as the remaining observation period after the 1st prolonged LH pulse interval. The effects of pharmacological agents on overall LH release were calculated by comparing the AUC in two consecutive 2-h post-treatment periods

with that in the 2-h pre-treatment period, using SigmaPlot version 11 (Systat Software). Statistical significance was tested using one-way ANOVA followed by Dunnett's test. All data were shown as mean ± S.E.M. $P < 0.05$ was considered statistically significant.

6.4 Results

6.4.1 Experiment 1: Effects of RFRP-3 on pulsatile LH release in OVX rats

To investigate the effects of RFRP-3 on pulsatile LH release, we examined the effects of centrally administered RFRP-3 on LH secretion in OVX rats. Central administration (icv) of RFRP-3 induced a dose-dependent inhibition of pulsatile LH release in OVX rats (Fig. 6.2). Injections of 0.1 nmol RFRP-3 had no significant effects on the duration of the LH pulse interval (Figs. 6.2, B and E). However, 1 nmol RFRP-3 increased the duration of the LH pulse interval by 27% (2-h pre-injection *vs*. 1st 2-h post-injection: 23.8 ± 1.6 *vs*. 30.2 ± 1.6 min; $P < 0.05$; Figs. 6.2, C and E). At the dose of 5 nmol, RFRP-3 increased the duration of pulse interval by 70% (2-h pre-injection *vs*. 1st 2-h post-injection: 24.0 ± 1.1 *vs*. 40.8 ± 1.6 min; $P < 0.05$; Figs. 6.2, D and E). Compared with 1 nmol RFRP-3, 5 nmol RFRP-3 had a significantly ($P < 0.05$) greater inhibitory effect on the pulsatile LH release. Furthermore, 5 nmol RFRP-3 had no effects on the LH pulses amplitude (2-h pre-injection *vs.* 1st 2-h post-injection *vs.* 2nd 2-h

post-injection: 2.3 ± 0.3 *vs*. 2.7 ± 0.3 *vs*. 2.5 ± 0.2 ng/ml; P > 0.05). In the control group, vehicle (aCSF) did not affect the pulsatile LH release (Figs. 6.2, A and E).

6.4.2 Experiment 2: Effects of RF-9 on LH release in OVX rats

To investigate the effects of RF-9, a RFRP-3 antagonist, on pulsatile LH release, we examined the effects of centrally administered RF-9 on LH secretion in OVX rats. Central administration (icv) of RF-9 did not affect the pulsatile LH release, but resulted in a dose-dependent increase of overall LH secretion, calculated by AUC (Fig. 6.3). Injections of 0.1 nmol RF-9 had no significant effects on LH secretion (Figs. 6.3, B and F). However, 1 nmol RF-9 significantly increased LH secretion by 19% (AUC, 2-h pre-injection *vs*. 1st 2-h post-injection: 556.8 ± 15.7 *vs*. 661.8 ± 18.3 ng/ml.min; P < 0.05; Figs. 6.3, C and F). At the dose of 10 nmol, RF-9 increased the LH secretion by 42% (AUC, pre-injection *vs*. 1st 2-h post-injection: 560.2 ± 19.0 *vs*. 793.4 ± 45.6 ng/ml.min; P < 0.05) and this increase was more potent (P < 0.05) than that of 1 nmol RF-9 (Figs. 6.3, D and F). In the control group, vehicle (aCSF) had no effects on LH secretion (Figs. 6.3, A and F). Neither of the above doses of RF-9 significantly affected LH pulse interval, even with the highest dose (10 nmol) of RF-9 (2-h pre-injection *vs*. 1st 2-h post-injection: 23.0 ± 1.2 *vs*. 26.8 ± 1.9; P > 0.05; Fig. 6.3, E). Furthermore,

10 nmol RF-9 had no effects on the amplitude of LH pulses (2-h pre-injection *vs.* 1st 2-h post-injection *vs.* 2nd 2-h post-injection: 2.5 ± 0.2 *vs*. 2.1 ± 0.3 *vs*. 2.2 ± 0.3 ng/ml; P > 0.05).

6.4.3 Experiment 3: Effects of RFRP-3 on pulsatile LH release in the presence of RF-9 in OVX rats

To investigate whether RFRP-3 induced suppression of pulsatile LH release is through the receptor GRP147, RFRP-3 (5 nmol) was administered (icv) into rats in the presence of RF-9 (30 nmol, icv). The RFRP-3-induced inhibition of pulsatile LH secretion was blocked by co-administration of RF-9 (LH pulse interval in 1st 2-h post injection, vehicle + RFRP-3 *vs*. RF-9 + RFRP-3: 40.4 ± 1.8 vs. 27.0 ± 1.4 min; P < 0.05; Figs. 6. 4, B, D and E). Similar to its single injection, three sequential injections of 10 nmol RF-9 *per se* did not affect the pulsatile LH release (LH pulse interval, 2-h pre-injection *vs*. 1st 2-h post-injection *vs*. 2nd 2-h post-injection: 22.9 ± 1.1 *vs*. 26.2 ± 1.6 *vs*. 24.2 ± 0.8 min; P > 0.05; Figs. 6. 4, C and E). Furthermore, three sequential injections of vehicle (aCSF) had no effects on the pulsatile LH release in control rats (Figs. 6. 4, A and E). Like the single injection, three sequential injections of RF-9 (10 nmol) increased the overall circulating levels of LH, calculated by AUC (pre-injection *vs*. 1st 2-h post-injection: 571.3 ± 16.1 *vs*. 769.7 ± 37.6 ng/ml.min; P < 0.05). RFRP-3 significantly suppressed overall LH secretion (AUC,

pre-injection *vs*. 1st 2-h post-injection: 590.1 ± 13.3 *vs*. 498.9 ± 21.2 ng/ml.min; $P < 0.05$), and this suppression was blocked by co-administration of RF-9 (AUC in the 1st 2-h post-injection, vehicle + RFRP-3 *vs*. RF-9 + RFRP-3: 498.9 ± 21.2 *vs*. 564.2 ± 19.1 ng/ml.min; $P < 0.05$).

6.4.4 Experiment 4: Effects of RF-9 on CRF or stress-induced inhibition of pulsatile LH release in OVX rats

To investigate the role of RFRP-3 in the stress-induced inhibition of pulsatile LH release, we administered CRF (2 nmol, icv), a central component of stress response, to OVX rats in the presence of RF-9 (30 nmol, icv). Furthermore, we exposed rats to two different stressors (restraint and LPS) in the presence of RF-9 (30 nmol, icv) (Fig. 6.5).

Central administration of CRF inhibited the pulsatile LH release in OVX rats (LH pulse interval, 2-h pre-injection *vs*. 1st 2-h post-injection: 24.3 ± 0.5 *vs*. 44.4 ± 2.4 min; $P < 0.05$; Figs. 6.5, B and D). This inhibitory effect was significantly attenuated by co-administration of RF-9 (LH pulse interval in 1st 2-h post-injection, vehicle + CRF *vs*. RF-9 + CRF: 44.4 ± 2.4 *vs*. 31.8 ± 4.9 min; $P < 0.05$), but this blockage was partial (Figs. 6.5, B, C and D). Control rats had no effects on the pulsatile LH release (Figs. 6.5, A and D)

Restraint stress induced an immediate suppression of pulsatile LH release in OVX rats (LH pulse interval, 2-h pre-injection *vs*. 1st prolonged interval

post-injection: 22.9 ± 0.5 *vs*. 49.7 ± 3.5 min; $P < 0.05$; Figs. 6.5, F and H). This inhibitory effect was significantly attenuated by co-administration of RF-9 (1st prolonged interval post-injection, vehicle + restraint *vs*. RF-9 + restraint: 49.7 ± 3.5 *vs*. 34.1 ± 3.9 min; $P < 0.05$), even though this blockage was partial (Figs. 6.5, F, G and H). Similarly, control rats had no effects on the pulsatile LH release (Figs. 6.5, E and H).

Administration of LPS evoked a profound inhibitory effect on the pulsatile LH release with the 1st prolonged LH pulse interval lasting over 2 h in OVX rats (1st prolonged interval post-injection, vehicle + vehicle *vs*. vehicle + LPS: 23.2 ± 1.0 *vs*. 138.8 ± 17.2 min; $P < 0.05$; Figs. 6.5, J and L). An inhibitory effect was also found in the recovery period (LH pulse interval in the recovery period, vehicle + vehicle *vs*. vehicle + LPS: 23.1 ± 1.2 *vs*. 40.7 ± 3.2 min; $P < 0.05$; Figs. 6.5, J and L). Co-administration of RF-9 decreased LPS-induced 1st prolonged LH pulse interval (1st prolonged interval post-injection, vehicle + LPS *vs*. RF-9 + LPS: 138.8 ± 17.2 *vs*. 78.3 ± 13.0 min; $P < 0.05$), even though this blockage was partial (Figs. 6.5, J, K and L). Furthermore, RF-9 totally blocked the inhibitory effects of LPS on the pulsatile LH release in the recovery period (Figs. 6.5, K and L). Control rats had no effects on the pulsatile LH release (Figs. 6.5, I and L).

6.4.5 Experiment 5: Effects of opioid receptor antagonists on RFRP-3-induced inhibition of pulsatile LH release in OVX rats

To investigate whether the opioid peptides signalling systems are involved in RFRP-3-induced inhibition of pulsatile LH secretion, we administered RFRP-3 (5 nmol, icv) to OVX rats pre-treated (icv) with naloxone (30 nmol), nor-BNI (6.8 nmol) or naltrindole (12 nmol). The RFRP-3-induced inhibition of pulsatile LH release was attenuated by co-administration of naloxone (1^{st} 2-h post-injection, vehicle + RFRP-3 *vs*. naloxone + RFRP-3: 41.1 ± 2.4 *vs*. 31.6 ± 2.1 min; $P < 0.05$; Figs. 6.6, E, F and I). However, RFRP-3-induced inhibition of pulsatile LH secretion was not affected by pre-treatment of nor-BNI or naltrindole (1^{st} 2-h post-injection, vehicle + RFRP-3 *vs*. nor-BNI + RFRP-3 *vs*. naltrindole + RFRP-3: 41.1 ± 2.4 *vs*. 38.3 ± 2.6 *vs*. 39.6 ± 2.5 min; $P > 0.05$; Figs. 6.6, E, G, H and I). Furthermore, administration of opioid receptor antagonists alone with vehicle had no effects on the pulsatile LH secretion (Figs. 6.6, A-D).

6.4.6 Experiment 6: Effects of intra-ARC administration of RFRP-3 or RF-9 on LH release in OVX rats with or without E_2 replacement

To investigate the role of RFRP-3 signalling in the ARC on LH release in the presence of different E_2 levels, we examined the effects of intra-ARC administration of RFRP-3 or RF-9 on LH secretion in both OVX+0×E_2 and

OVX+2×E_2 rats (Fig. 6.7).

Intra-ARC administration of RFRP-3 (500 pmol) or RF-9 (1 nmol) had no effects on pulsatile LH release in OVX+0×E_2 rats (LH pulse interval in RFRP-3 group, 2-h pre-injection *vs.* 1st 2-h post-injection *vs.* 2nd 2-h post-injection: 22.6 ± 1.2 *vs*. 24.4 ± 1.2 *vs*. 23.0 ± 1.1 min; P > 0.05; LH pulse interval in RF-9 group, 2-h pre-injection *vs.* 1st 2-h post-injection *vs.* 2nd 2-h post-injection: 23.7 ± 0.8 *vs*. 26.3 ± 1.4 *vs*. 22.9 ± 1.1 min; P > 0.05; Figs. 6.7, B, C, and G). Furthermore, RFRP-3 and RF-9 had no effects on the LH pulse amplitude. Accordingly, both RFRP-3 and RF-9 did not affect the overall LH release (measured by AUC) in OVX+0×E_2 rats (Figs. 6.7, B, C and H). In the control group, intra-ARC administration of vehicle (aCSF) did not affect LH pulse frequency or overall LH secretion in OVX+0×E_2 rats (Fig. 6.7, A, G and H).

Intra-ARC administration of both vehicle (aCSF) and RFRP-3 (500 pmol) had no effects on overall LH release (measured by AUC) in OVX+2×E_2 rats (Figs. 6.7, D, E and H). However, LH secretion was significantly increased by intra-ARC administration of RF-9 (1 nmol) by nearly 1 fold in the 1st 2-h post-injection period (AUC, 2-h pre-injection *vs*. 1st 2-h post-injection: 301.0 ± 24.3 *vs*. 585.9 ± 50.9 ng/ml.min; P < 0.05; Figs. 6.7, F and H). This increase recovered to normal in the 2nd 2-h post-injection period (AUC, 2-h pre-injection *vs*. 1st 2-h post-injection: 301.0 ± 24.3 *vs*. 389.0 ± 24.6 ng/ml.min; P > 0.05; Figs. 6.7, F and H). In addition, no misplaced ARC cannulae were found in this set of experiments (Fig. 6.8).

6.4.7 Experiment 7: Effects of intra-mPOA administration of RFRP-3 or RF-9 on LH release in OVX rats with or without E_2 replacement

To investigate the role of RFRP-3 signalling in mPOA in LH release in different E_2 levels, we examined the effects of intra-mPOA administration of RFRP-3 or RF-9 on LH secretion in both OVX+0×E_2 and OVX+2×E_2 rats.

Intra-mPOA administration of RFRP-3 (500 pmol) had no effects on pulsatile LH release in OVX+0×E_2 rats (LH pulse interval, 2-h pre-injection *vs.* 1st 2-h post-injection *vs.* 2nd 2-h post-injection: 23.0 ± 1.9 *vs*. 24.5 ± 1.0 *vs*. 24.7 ± 1.9 min; P > 0.05; Figs. 6.9, B and G). Accordingly, RFRP-3 did not affect the overall LH release (calculated by AUC) in OVX+0×E_2 rats (Figs. 6.9, B and H). Interestingly, intra-mPOA administration of RF-9 (1 nmol) did not affect the LH pulse frequency in OVX+0×E_2 rats (LH pulse interval, 2-h pre-injection *vs.* 1st 2-h post-injection *vs.* 2nd 2-h post-injection: 23.7 ± 1.0 *vs*. 26.3 ± 1.4 *vs*. 26.1 ± 1.7 min; P > 0.05; Figs. 6.9, C and G). However, RF-9 increased the LH pulse amplitude in both the 1st and 2nd post-injection periods (LH pulse amplitude, 2-h pre-injection *vs.* 1st 2-h post-injection *vs.* 2nd 2-h post-injection: 2.0 ± 0.1 *vs*. 3.5 ± 0.5 *vs*. 4.1 ± 0.1 ng/ml; P < 0.05), and therefore increased the overall LH secretion in that 4-h period (AUC, 2-h pre-injection *vs*. 1st 2-h post-injection *vs*. 2nd 2-h post-injection: 550.8 ± 20.7 *vs*. 740.3 ± 15.4 *vs*. 760.1 ± 47.4 ng/ml.min;

$P < 0.05$; Figs. 6.9, C and H). Additional, in the control group, vehicle (aCSF) did not affect LH pulse frequency or overall LH secretion in OVX+0×E_2 rats (Figs. 6.9, A, G and H).

In OVX+2×E_2 rats, intra-mPOA administration of RFRP-3 (500 pmol) did not affect the overall LH release (calculated by AUC; Figs. 6.9, E and H). However, intra-mPOA administration of RF-9 (1 nmol) significantly increased the overall LH release by 1 fold in both the 1st and 2nd 2-h post-infusion periods (AUC, 2-h pre-injection *vs*. 1st 2-h post-injection *vs*. 2nd 2-h post-injection: 332.3 ± 18.3 *vs*. 620.9 ± 42.6 *vs*. 614.9 ± 60.8 ng/ml.min; $P < 0.05$; Figs. 6.9, F and H). In addition, the mPOA cannulae were found to be misplaced in two OVX+2×E_2 rats (Fig. 6.10). RF-9 (1 nmol) did not affect the LH secretion in those rats (AUC, 2-h pre-injection *vs*. 1st 1-h post-injection *vs*. 2nd 1-h post-injection: 332.3 ± 18.3 *vs*. 360.9 ± 32.6 *vs*. 344.9 ± 20.8 ng/ml.min, $P > 0.05$). Intra-mPOA administration of vehicle (aCSF) had no effects on overall LH release in OVX+2×E_2 rats (Figs. 6.9, D and H).

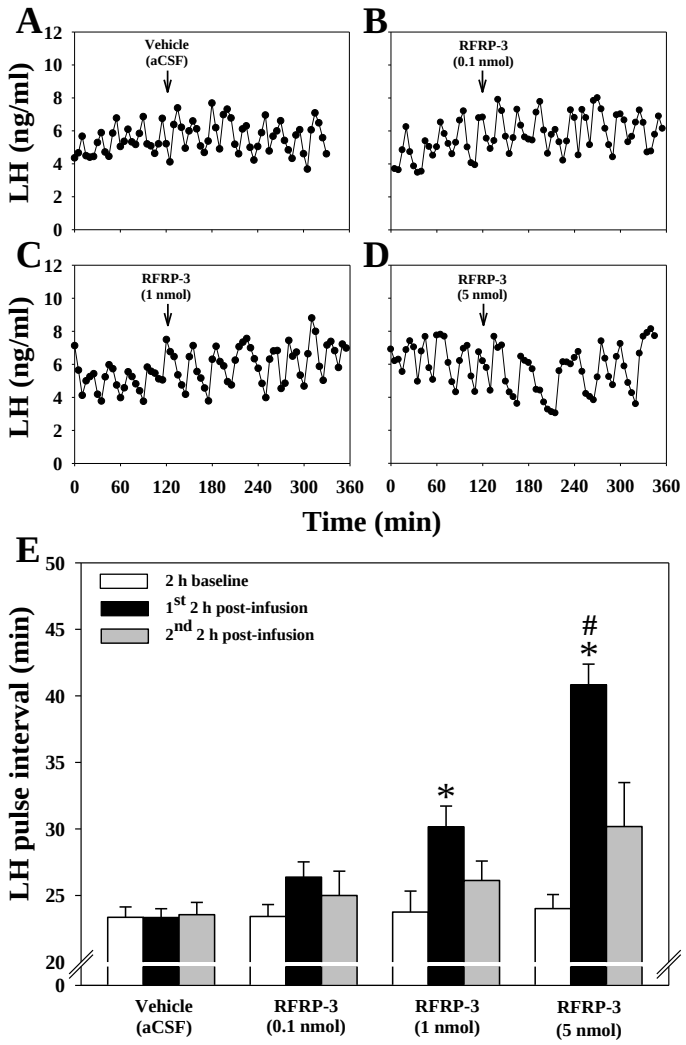

Fig. 6.2 Effects of RFRP-3 on pulsatile LH release in OVX rats. Representative LH profiles illustrating the effect of icv administration (arrow) of RFRP-3 (B-D), or vehicle (aCSF) (A) in OVX rats. Central administration of RFRP-3 resulted in a dose-dependent inhibition of pulsatile LH secretion, as summarised in panel E. *, $P < 0.05$ *vs.* 2-h baseline period within the same group, as well as the same 2-h period within the vehicle treated group; #, $P < 0.05$ *vs.* same 2-h period within the group treated with 1 nmol RFRP-3 (n = 5-8 per group).

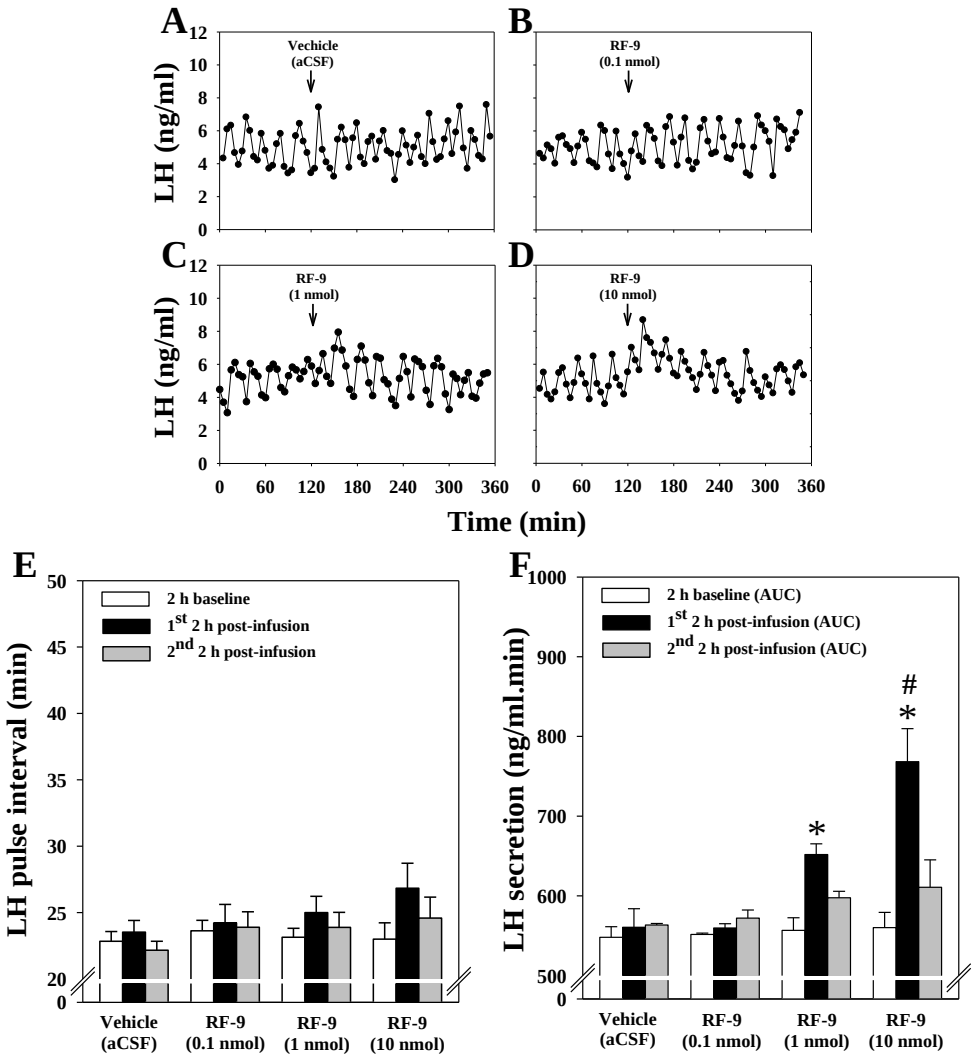

Fig. 6.3 Effects of RF-9 on LH release in OVX rats. Representative LH profiles illustrating effects of icv administration (arrow) of RF-9 (B-D), or vehicle (aCSF) (A) in OVX rats. Central administration of RF-9 had no significant effects on pulsatile LH secretion in OVX rats, as summarised in panel E, while resulted in a dose-dependent increase of LH secretion, calculated by AUC, as summarised in panel F. *, $P < 0.05$ *vs.* 2-h baseline period within the same group, as well as the same 2-h period within the vehicle treated group; #, $P < 0.05$ *vs.* same 2-h period within the group treated with 1 nmol RF-9 (n = 5-7 per group).

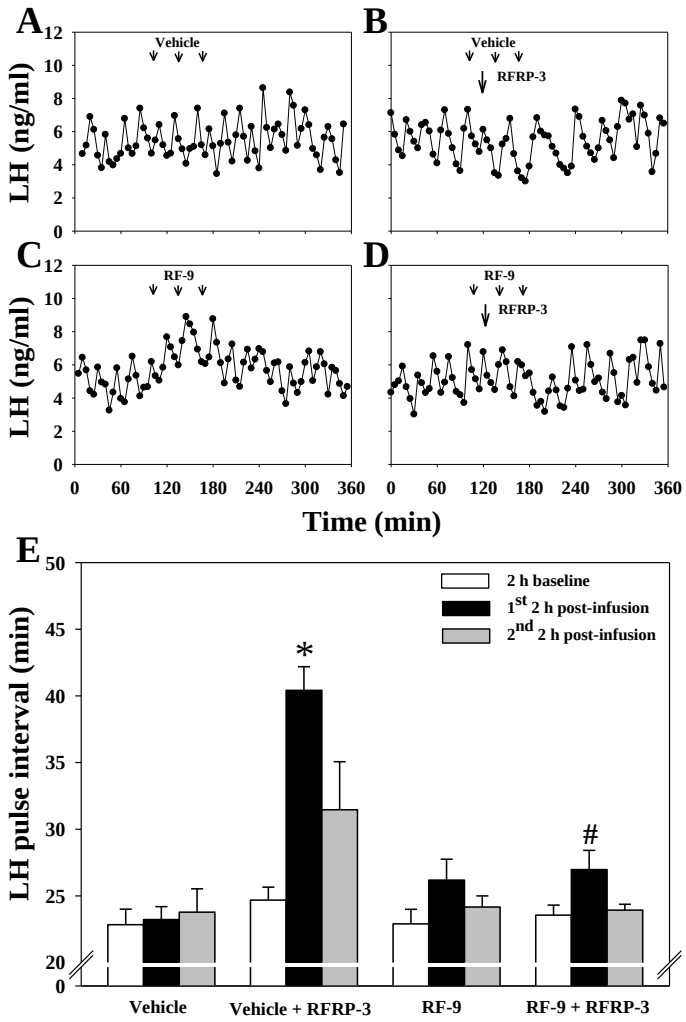

Fig. 6.4 Effects of RFRP-3 on pulsatile LH release in the presence of RF-9 in OVX rats. Representative LH profiles illustrating the effect of icv administration of 5 nmol RFRP-3 (arrow) in the presence of icv administration of 3×10 nmol RF-9 (D) (↓↓↓) or vehicle (aCSF) (B) (↓↓↓), as well as that of 3×10 nmol RF-9 (C) (↓↓↓) or vehicle (aCSF) (A) (↓↓↓) alone, in OVX rats. The RFRP-3-induced inhibition of pulsatile LH secretion was blocked by pre-treatment of RF-9, as summarised in panel E. *, $P < 0.05$ *vs.* 2-h baseline period within the same group, as well as the same 2-h period within the vehicle treated group; #, $P < 0.05$ *vs.* same 2-h period within the group treated with vehicle + RFRP-3 (n = 6-8 per group).

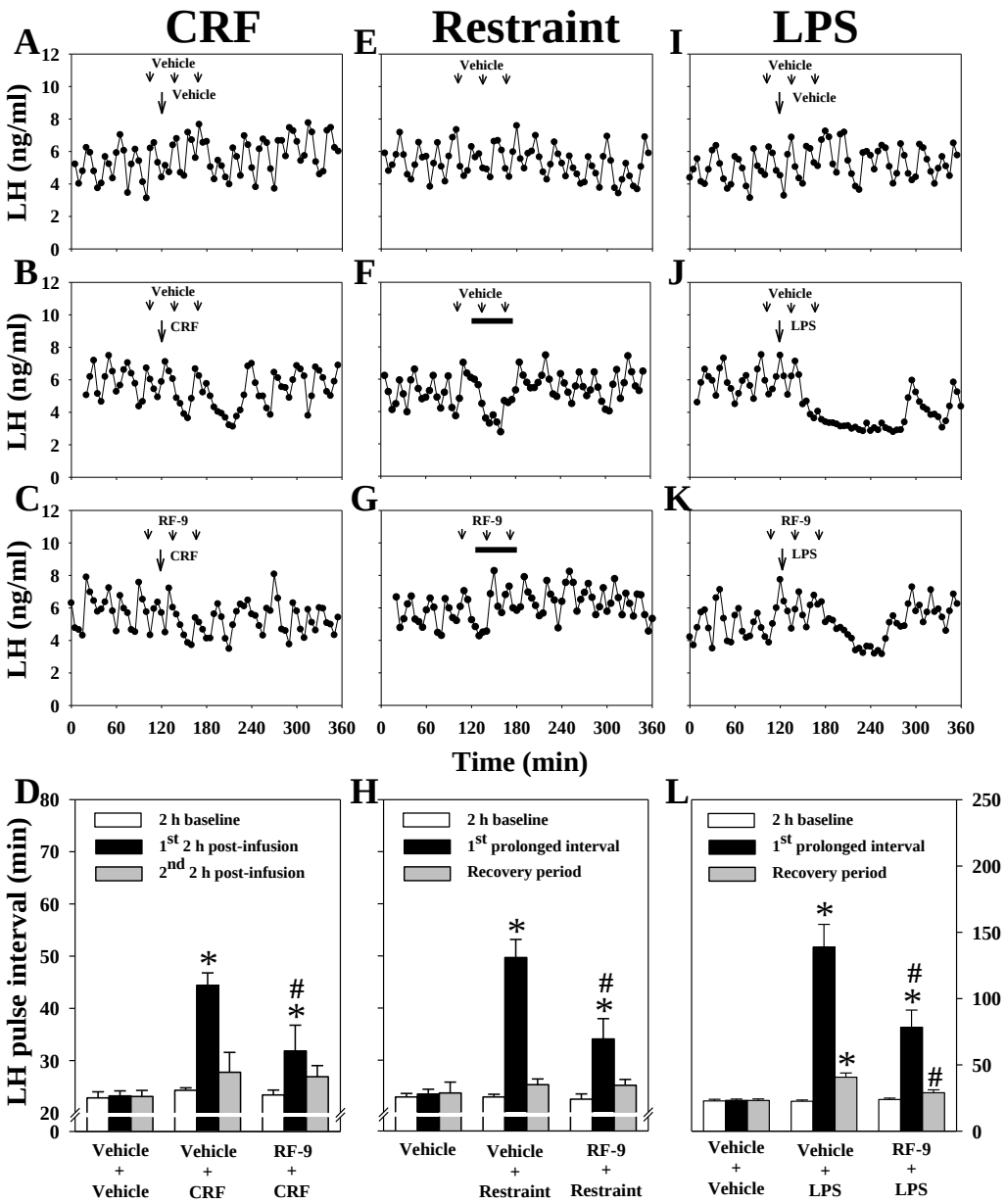

Fig. 6.5 Effects of RF-9 on CRF or stress-induced inhibition of pulsatile LH release in OVX rats. Representative LH profiles illustrating the effect of icv administration of 2 nmol CRF (arrow), applying of restraint (1 h) (horizontal bar) or iv administration of LPS (15 μg/kg) (arrow) in the presence of icv administration of 3×10 nmol RF-9 (↓↓↓) (C, G, K) or vehicle (↓↓↓) (B, F, J), as well as that of vehicle (A, E, I) alone, in OVX rats. RF-9 partially blocked the CRF, restraint, or LPS-induced inhibition of pulsatile LH secretion, as summarised in panel D, H, L. *, $P < 0.05$ *vs.* 2-h baseline period within the same group, as well as the same 2-h or 1st prolongation period within the vehicle treated group; #, $P < 0.05$ *vs.* same 2-h period, 1st prolongation period, or recovery period within the group treated with CRF or stress in the presence of vehicle (n = 5-9 per group).

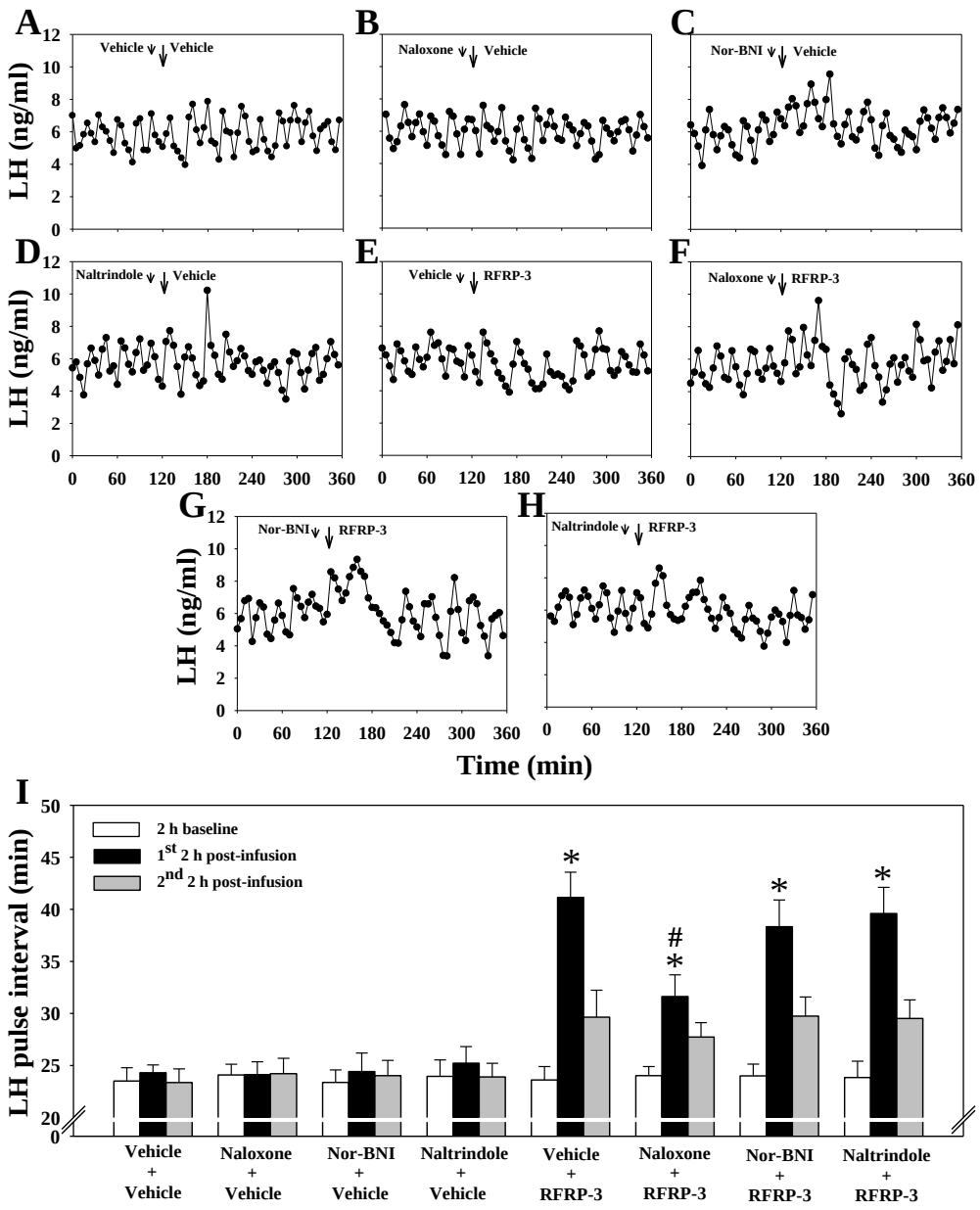

Fig. 6.6 Effects of opioid receptor antagonists on RFRP-3-induced inhibition of pulsatile LH release in OVX rats. Representative LH profiles illustrating effects of icv administration (arrow) of 5 nmol RFRP-3 in the presence of icv administration (↓) of specific opioid receptor antagonists [μ-receptor antagonist: 30 nmol naloxone, (F); κ-receptor antagonist: 6.8 nmol nor-BNI, (G); δ-receptor antagonist: 12 nmol naltrindole, (H)] or vehicle (aCSF) (E), as well as that of endogenous opioid receptor antagonists (same dose as above) alone (B-D), in OVX rats. Naloxone partially blocked the RFRP-3-induced inhibition of pulsatile LH secretion, as summarised in panel I. *, $P < 0.05$ *vs.* 2-h baseline period within the same group, as well as the same 2-h period within the vehicle treated group; #, $P < 0.05$ *vs.* same 2-h period within the group treated with RFRP-3 in the presence of vehicle, nor-BNI or naltrindole (n = 6-11 per group).

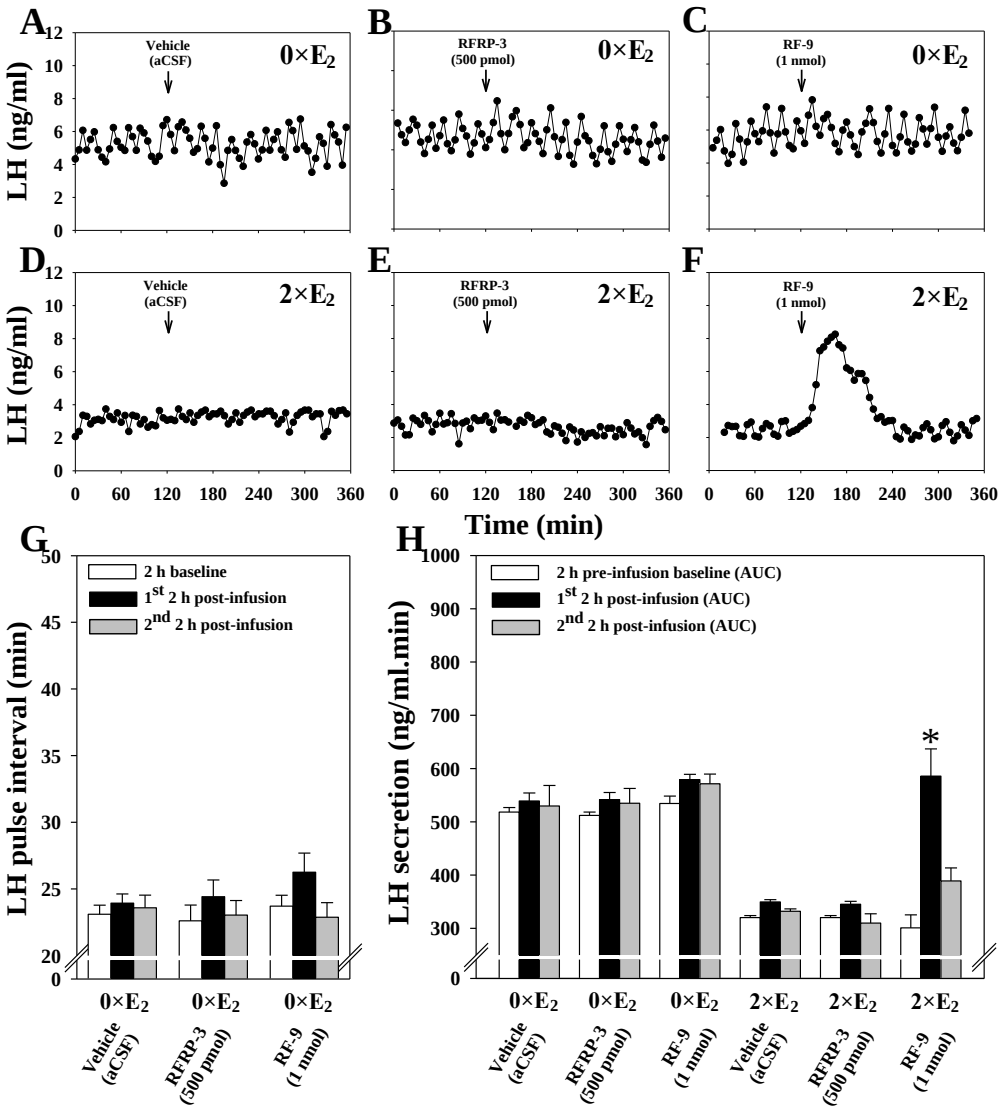

Fig. 6.7 Effects of intra-ARC administration of RFRP-3 or RF-9 on LH release in OVX rats with or without E_2 replacement. Representative LH profiles demonstrating (1) effects of intra-ARC administration (arrow) of vehicle (aCSF), RFRP-3 or RF-9 on pulsatile LH secretion in OVX rats without E_2 capsule replacement (labelled as $0\times E_2$) (A-C), and (2) effects of intra-ARC administration (arrow) of vehicle (aCSF), RFRP-3 or RF-9 on LH secretion in OVX rats replaced with two E_2 capsules (labelled as $2\times E_2$) (D-F). Intra-ARC administration of both RFRP-3 and RF-9 had no effects on the pulsatile LH release in OVX+$0\times E_2$ rats, as summarised in panel G. Furthermore, intra-ARC administration of RF-9 stimulated LH secretion in OVX+$2\times E_2$ rats, but not in OVX+$0\times E_2$ rats, as summarised in panel H. *$P < 0.05$ *vs.* 2-h baseline within the same group, as well as the same 2-h period within group treated with corresponding vehicle (n = 4-8 per group).

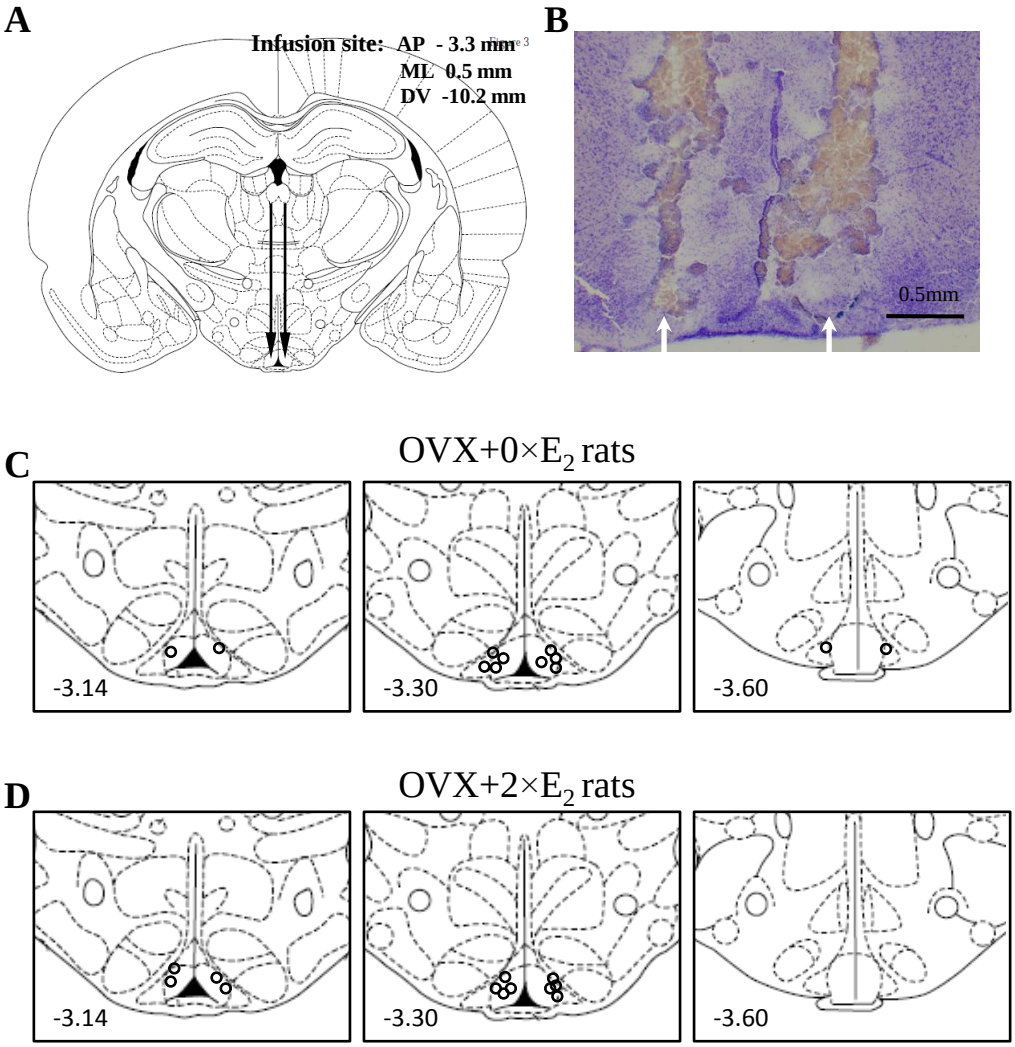

Fig. 6.8 Schematic illustration and photomicrograph of the infusion site targeted to the ARC. A, schematic illustration showing the target site for bilateral cannulation of the ARC. Arrows point to the location of the tips of internal cannulae. B, photomicrograph of a coronal brain section in a representative animal implanted with a bilateral cannula in the ARC. Arrows indicate the tips of the internal cannulae. C-D, schematic drawings of the ARC illustrating the individual sites of injection for the experiments carried out in OVX+0×E_2 and OVX+2×E_2 rats, respectively. Numbers in each drawing indicate the distance (mm) to Bregma. Open circles show the right injection sites.

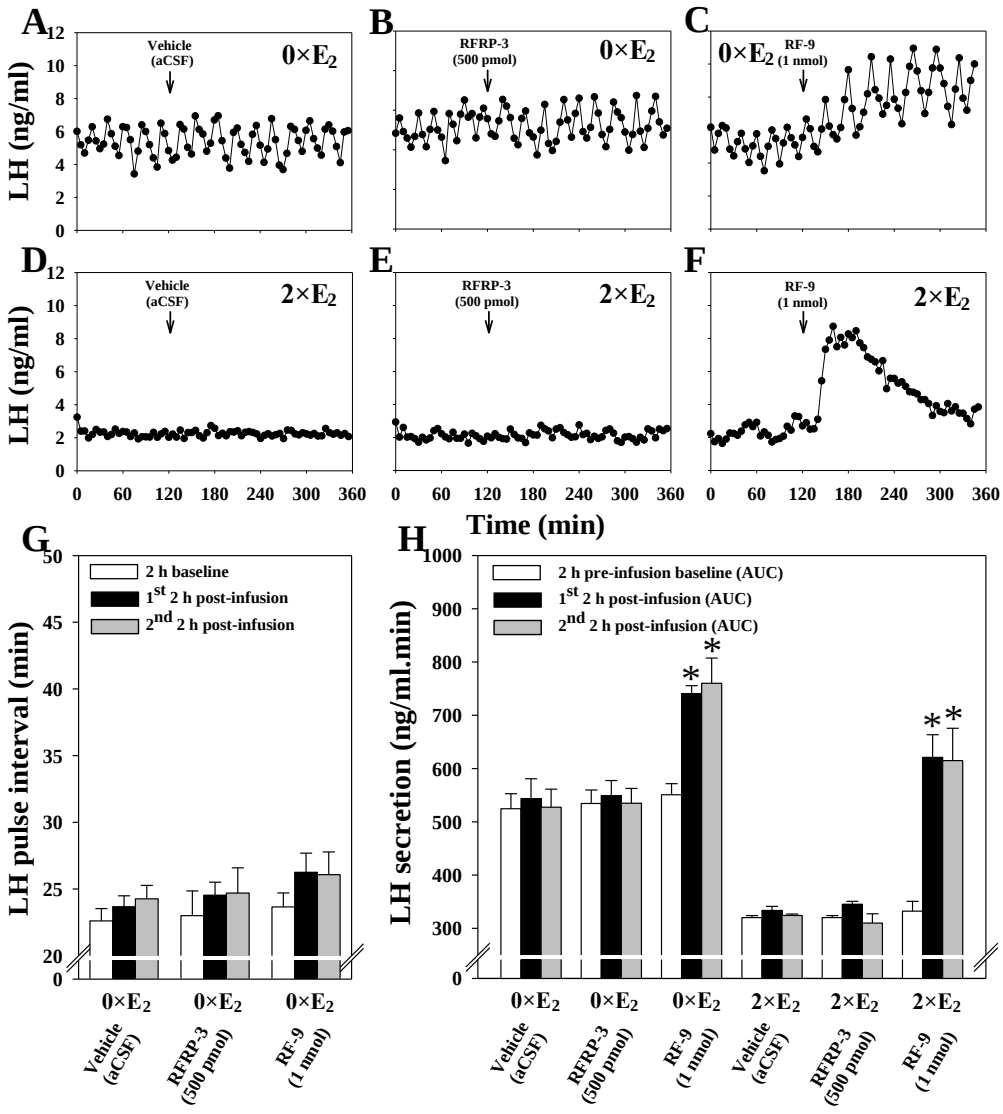

Fig. 6.9 Effects of intra-mPOA administration of RFRP-3 or RF-9 on LH release in OVX rats with or without E_2 replacement. Representative LH profiles demonstrating (1) effects of intra-mPOA administration (arrow) of vehicle (aCSF), RFRP-3 or RF-9 on pulsatile LH secretion in OVX rats without E_2 capsule replacement (labelled as $0\times E_2$) (A-C), and (2) effects of intra-mPOA administration (arrow) of vehicle (aCSF), RFRP-3 or RF-9 on LH secretion in OVX rats replaced with two E_2 capsules (labelled as $2\times E_2$) (D-F). Intra-mPOA administration of both RFRP-3 and RF-9 had no effects on the pulsatile LH release in OVX+$0\times E_2$ rats, as summarised in panel G. However, intra-mPOA administration of RF-9 stimulated LH secretion in both OVX+$0\times E_2$ and OVX+$2\times E_2$ rats, as summarised in panel H. *$P < 0.05$ *vs.* 2-h baseline within the same group, as well as the same 2-h period within the group treated with corresponding vehicle (n = 4-7 per group).

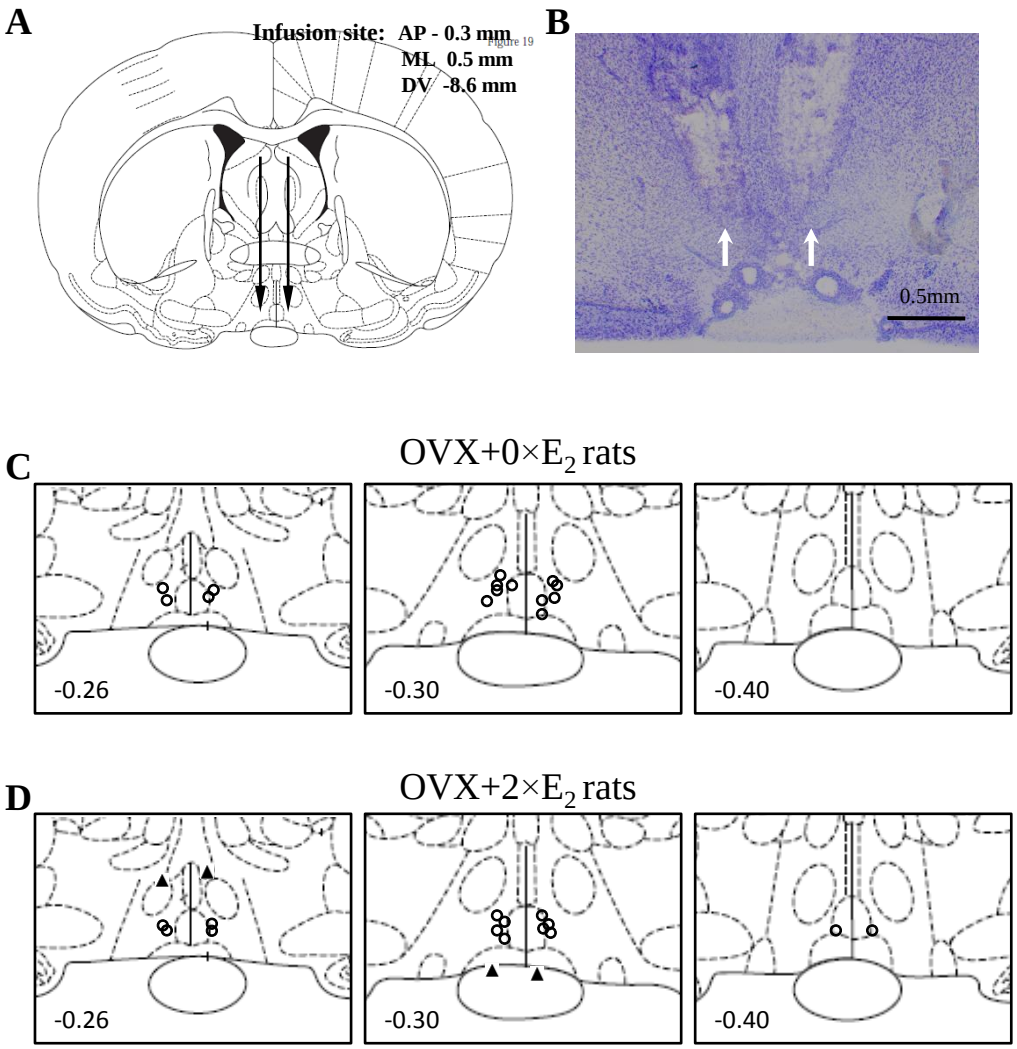

Fig. 6.10 Schematic illustration and photomicrograph of the infusion site targeted to the mPOA. A, schematic illustration showing the target site for bilateral cannulation of the mPOA. Arrows point to the location of the tips of internal cannulae. B, photomicrograph of a coronal brain section in a representative animal implanted with a bilateral cannula in the mPOA. Arrows indicate the tips of the internal cannulae. C-D, schematic drawings of the mPOA illustrating the individual sites of injection for the experiments carried out in OVX+0×E_2 and OVX+2×E_2 rats, respectively. Numbers in each drawing indicate the distance (mm) to Bregma. Open circles show the right injection sites. Close triangles show the misplaced injection sites.

6.5 Discussion

In the present study we provide evidence to suggest that RFRP-3 signalling regulates the frequency of the GnRH pulse generator, since central administration of RFRP-3 via icv cannulae induced a dose-dependent inhibition of pulsatile LH release in OVX rats. This is in agreement with previous studies that showed that central administration (icv) of 5 nmol RFRP-3 induced a suppressive effect on mean LH secretion in OVX rats (Pineda *et al.* 2010b). It is also in keeping with several other studies showing inhibitory effects of central administered RFRP-3 on mean LH release in rodents (Johnson *et al.* 2007, Leon *et al.* 2014). However, the present study is the first to indicate that these effects may relate to the inhibition of GnRH pulse generator frequency. This inhibitory effect on pulsatile LH release was not shown in previous studies that monitored LH pulses (Murakami *et al.* 2008, Anderson *et al.* 2009). These studies used comparable doses of RFRP-3 to those of the current study, however, no effects on the LH pulse frequency, amplitude or mean LH concentration were reported (Murakami *et al.* 2008, Anderson *et al.* 2009), which is at odds with the inhibitory role of RFRP-3 in the HPG axis. This may be due to the differences in experimental animal strains or gonadal steroid milieu. Murakami *et al.* (2008) used OVX Wistar rats and Anderson *et al.* (2009) used OVX Sprague-Dawley rats with E_2 replacement, whereas we used OVX Sprague-Dawley rats without E_2 replacement. Furthermore, we collected blood samples more frequently and for a longer period, which may have helped to reveal the modest inhibitory effect

of RFRP-3 as shown in the present study.

Central administration of RF-9 *per se* increased LH secretion (measured by AUC) in OVX rats in a dose-dependent fashion, which is in keeping with previous studies (Pineda *et al.* 2010c). Furthermore, the present study has shown that RF-9 did not affect the frequency of LH pulses, at least not with the doses used. This unchanged LH pulse frequency by central administered RF-9 in OVX rats may indicate a lack of endogenous RFRP-3 tone on the GnRH pulse generator in hypoestrogenic conditions. Furthermore, we have shown that RF-9 blocks the inhibitory effects of RFRP-3 on pulsatile LH release in OVX rats. Since RF-9 was identified as one of GPR147 receptor antagonist (Hinuma *et al.* 2000, Pineda *et al.* 2010c), it indicates that exogenous RFRP-3 act on GPR147 receptor to suppress pulsatile LH release. This is in keeping with recent studies showing that the inhibitory effects of RFRP-3 on LH release are abrogated in *GPR147* knockout mice (Leon *et al.* 2014). However, the present study further implicates the GnRH pulse generator as the underlying mechanism for this blockade effect. Of note, RF-9 was reported as a potent and selective antagonist of receptors for both RFRPs and neuropeptide FF, a related peptide in the same family of RFRPs. Since central administration of RFRP-1 and neuropeptide FF had no effects on LH secretion in OVX rats (Pineda *et al.* 2010b), the stimulatory effects of RF-9 were likely mediated through the blockade of RFRP-3's actions. However, a recent study from Allan Herbison's group reported that RF-9 could directly excite the electrical activity of GnRH neurons,

and that this stimulation was not observed in *Kiss1r* knockout mice (Liu and Herbison 2014). It is therefore possible that RF-9 function as a GPR147 antagonist, as well as a Kiss1r agonist on GnRH neurones. This is supported by another more recent study showing that the stimulatory effect of RF-9 on LH release was significantly blunted in *Kiss1r* knockout mice and selective rescue of *Kiss1r* expression in GnRH neurones restored the stimulatory effect of RF-9 on LH release (Min *et al.* 2015). Similarly, it has been shown that RF-9-induced LH secretion is blocked by a Kiss1r antagonist in female rats (Sahin *et al.* 2015). Therefore, substantial evidence has accumulated very recently to indicate that RF-9 can function as a Kiss1r agonist which will definitely complicate the interpretation for the findings of the present study. However, previous studies have shown that iv administered Kiss1 did not affect the frequency of the GnRH pulse generator monitored electrophysiologically by measurement of MUA volleys in the ARC of OVX rats, although it induced a concomitant heightened LH secretion (Kinsey-Jones *et al.* 2008). Therefore, activation of Kiss1r by RF-9, if possible, does not necessarily induce an increased LH pulse frequency.

The present study has also shown that central (icv) administration of RFRP-3 or RF-9 had no effect on the amplitude of LH pulses. LH pulse amplitude could be subject to regulations at various levels in the HPG axis. First, the amplitude of LH pulses can be affected by the frequency of the GnRH pulses due to the amount of releasable LH in the pituitary glands (Clarke and Cummins 1985). Therefore, high frequency GnRH pulses lead to low LH pulse amplitude,

whereas low frequency lead to high pulse amplitude (Clarke and Cummins 1985, Wu *et al.* 1987). Second, RFRP-3 may modulate the amplitude of GnRH pulses by acting directly on GnRH neurones. Indeed, an *in vitro* study showed that RFRP-3 could suppress the amplitude of GnRH pulses (Glanowska *et al.* 2014). Therefore, this inhibitory effect may abrogate the stimulatory effects on LH pulse amplitude induced by the decreased LH pulse frequency, which may provide an explanation for the unaltered LH pulse amplitude following central administration of RFRP-3 in the present study. The same rationale could apply to RF-9. Finally, these inhibitory/stimulatory effects of RFRP-3/RF-9 could be due to interactions at the level of pituitary. Previous studies have implicated RFRP-3 signalling in the regulation of gonadotrophs in anterior pituitary since peripheral injection (iv) of RFRP-3 inhibited, whereas RF-9 stimulated, LH secretion in rodents (Murakami *et al.* 2008, Pineda *et al.* 2010c, Poling *et al.* 2014). Furthermore, exposure to RFRP-3 suppressed LH secretion from pituitary explants of rodents (Pineda *et al.* 2010b, Leon *et al.* 2014). This suppression was due to the GPR147 receptor expressed in the pituitary, since it was blocked by co-administration of RF-9 and was absent in pituitary explants from *GPR147* knockout mice (Pineda *et al.* 2010b, Leon *et al.* 2014). However, it should be noted that RFRP-3 is unlikely to function as a hypophysiotrophic neuropeptide due to the absence of RFRP fibres in the external layer of ME (Kriegsfeld *et al.* 2006, Johnson *et al.* 2007, Rizwan *et al.* 2009). Nor does RFRP-3 function as a para/autocrine regulating neuropeptide, since only *GPR147* but not *RFRP*

mRNA was displayed in the pituitary (Hinuma *et al.* 2000). Therefore, the mechanisms underlying the regulation of RFRP-3 at the anterior pituitary in rodents remain to be elucidated.

We have also shown that RF-9 partially blocked both restraint (psychological stressor) and LPS (immunological stressor)-induced inhibition of pulsatile LH release. If these blocade effects are via the antagonism of GPR147, RFRP-3 might be involved in the suppression of the HPG axis by different stressors. This is in agreement with previous studies which showed that hypothalamic *Rfrp* mRNA expression was up-regulated in response to acute psychological and immunological stress in rats (Kirby *et al.* 2009, Iwasa *et al.* 2014). Other recent studies have also implicated RFRP-3 signalling in chronic psychological stress and metabolic stress-induced reproductive dysfunctions (Batool *et al.* 2014, Leon *et al.* 2014, Geraghty *et al.* 2015). The present study adds to our understanding of the underlying mechanism of RFRP-3 signalling to mediate stress-induced suppression of the HPG axis, by showing a modulation via the GnRH pulse generator. Furthermore, it should be noted that psychological stress did not affect *Rfrp* mRNA expression in sheep (Papargiris *et al.* 2011) and immunological stress did not affect *GnIH* mRNA expression in birds (Lopes *et al.* 2012). These data indicate that species differences may exist in the RFRPs/GnIH signalling systems in response to stress.

Stress-induced suppression of the HPG axis involves a variety of neuropeptides. It is well established that the CRF signalling system is a potent inhibitor of the

GnRH pulse generator and is involved in both LPS and restraint stress-induced suppression of pulsatile LH release (Li *et al.* 2010). Yet, the role of RFRP-3 signalling in mediating these stress responses is unknown. The present study has shown that CRF-induced suppression of LH can be reversed by the RFRP-3 antagonist. Therefore, RFRP-3 may be one of the downstream mediators of CRF-induced suppression of LH. Indeed, CRF-R1 are found on RFRPs neurones (Kirby *et al.* 2009) and central administration of CRF induced increased neuronal activity in DMH where RFRP-3 neurones are located (Imaki *et al.* 1993). Furthermore, different types of stressors activate specific CRF receptor subtypes. Whilst restraint-induced suppression of GnRH pulse generator frequency is mediated via both CRF-R1 and CRF-R2, metabolic and immunological stress are only mediated via CRF-R2 (Li *et al.* 2006). Therefore, it is postulated that CRF-R2 is also expressed in RFRP-3 neurones. Indeed, previous studies have shown that CRF-R2 was expressed in DMH (Van Pett *et al.* 2000). Furthermore, RF-9 only partially blocked the inhibitory effects induced by CRF as well as restraint and LPS stress. This is not surprising since the CRF signalling system may operate through other downstream mediators such as GABA and endogenous opioid peptides to affect pulsatile LH release (Li *et al.* 2010).

The present study has shown that the μ-opioid receptor mediates, at least in part, the effect of RFRP-3 on the pulsatile LH release since opioid antagonist naloxone partially blocks RFRP-3-induced suppression of LH pulse frequency. It

was also shown that antagonism of κ-receptor with nor-BNI or δ-receptor with naltrindole had no effects on RFRP-3-induced inhibition of pulsatile LH release. This is in keeping with a previous study that showed only μ-opioid receptor antagonist partially blocked the GnIH-induced food intake in chickens, but not with κ- and δ-receptor antagonists (Tachibana *et al.* 2008). Thus, it is tempting to speculate that endogenous opioid peptides may modulate various physiological or pharmacological traits of the GnIH/RFRP-3 signalling system, and this modulation may be via the μ-opioid receptor which is expressed on GnRH neurones (Zheng *et al.* 2005). It has been suggested that among the various endogenous opioid peptides in the brain, β-endorphin show high affinity for the μ-opioid receptor (Tachibana *et al.* 2008). Furthermore, β-endorphin is involved in the inhibitory effects of pulsatile GnRH secretion (Ogata *et al.* 2009). Therefore, it is possible that RFRP-3 neurones may project to β-endorphin containing neurones. As shown in the present study, CRF-induced suppression of LH release was also blocked by RFRP-3 antagonist. Furthermore, CRF or stress-induced suppression of LH release can also be reversed by naloxone (Gindoff and Ferin 1987, O'Byrne *et al.* 1989), which indicates that endogenous opioid peptides may be the downstream effectors of both CRF/stress and RFRP-3 signalling systems. Taken together, it is reasonable to speculate that CRF, RFRP-3 and endogenous opioid neuropeptides may form a sequential link to modulate stress-induced suppression of LH secretion.

We further investigated the specific locations for RFRP-3 signalling to exert its

inhibitory effects on the pulsatile LH release. Administration of RFRP-3 in the ARC or mPOA was unable to affect the pulsatile release of LH in OVX+0×E_2 rats. Administration of RF-9 in the ARC or mPOA did not affect the frequency of LH pulses in OVX+0×E_2 rats, even though RF-9 in the mPOA induced an increase of overall LH secretion measured by AUC. Likewise, a previous study in sheep showed that icv administration of RF-9 induced a dramatic increase in LH release, but icv administration of RFRP-3 had no effects on LH release (Caraty *et al.* 2012). A possible explanation for this discrepancy could be the potential action of RF-9 on Kiss1r, as discussed above. Indeed, it has been shown that intra-mPOA administration of Kiss1 induced a robust LH secretion in OVX rats (Li *et al.* 2009b). However, the dynamics of stimulated LH secretion are quite different: Kiss1 action in the mPOA induced an immediate surge-like secretion of LH, whereas RF-9 elicited a more gradual and sustained increase in the amplitude of LH pulses which lasted more than 4 h after the intra-mPOA injection. The present study has also shown that RFRP-3 signalling in the ARC was not involved in the pulsatile LH release. It indicated that the suppression of pulsatile release of LH by icv administered RFRP-3, as shown in the present study, may not involve the GnRH pulse generator in the ARC directly. Although morphological studies have implicated RFRP-3 neurones via direct interactions with Kiss1 neurones in the ARC (postulated to be one component of the GnRH pulse generator) (Poling *et al.* 2013), RFRP-3 may be able to mediate its effects on the GnRH pulse generator indirectly through other signalling pathways, such

as endogenous opioid peptides. Taken together, the results of the present study dispute the ARC and mPOA as the site of action by which centrally administered RFRP-3 inhibits the pulsatile release of LH in OVX rats. This inhibitory effect may involve intermediate signalling systems which could eventually affect the GnRH secretion.

The present study has also shown that RF-9 acts in both the ARC and mPOA to induce a profound secretion of LH in OVX+2×E_2 rats. This indicates that RF-9 had a more robust stimulatory effect in the presence of raised E_2 levels, which is in accordance with the inhibitory role of RFRP-3 in E_2 negative feedback control of gonadotrophins (Kriegsfeld *et al.* 2006, Leon *et al.* 2014). The present study is in keeping with previous studies which showed that E_2 supplementation augmented the LH secretion induced by RF-9 in intact female rats (Pineda *et al.* 2010c). We further indicated that both the mPOA and ARC are involved in this augmentation induced by E_2. This is in keeping with the notion that inhibitory effects of E_2 on the HPG axis involve multiple brain sites including the mPOA and ARC (Herbison 1998). The present study has also shown that RFRP-3 administered into mPOA or ARC did not inhibit the LH release in OVX+2×E_2 rats. This may due to the enhanced endogenous RFRP-3 tone in these locations which could block any effects of exodogenous RFRP-3 signalling. Previous studies have shown that the expression of *RFRP* mRNA in the DMH did not vary between intact, OVX and OVX+E_2 rats (Quennell *et al.* 2010). Neither did the expression of *GPR147* mRNA in the mPOA or ARC fluctuate among these

groups (Quennell *et al.* 2010). It is therefore likely that the enhanced RFRP-3 inhibitory tone during E_2-induced negative feedback control may be due to the augmented RFRP-3 function in the mPOA and ARC.

In summary, the results of the present study provide evidence of a suppressive effect of RFRP-3 on pulsatile LH release in female rats. This effect may be mediated via endogenous opioid peptides. Furthermore, RFRP-3 antagonism partially blocks CRF/stress-induced suppression of pulsatile LH release, which indicates that RFRP-3 is an important stress modulator on the HPG axis. Finally, these data also suggest that RFRP-3 signalling in the mPOA and ARC may be involved in E_2-induced negative feedback regulation of LH secretion.

CHAPTER SEVEN: KISS1 AND $GABA_B$ RECEPTOR SIGNALLING IN THE MEA MODULATES LH SECRETION IN FEMALE RATS

7.1 Introduction

Extra-hypothalamic structures have long been indicated as important modulators of HPG axis. Among them, the amygdala has been implicated in regulating various reproductive functions, including ovulation, oestrous cyclicity, gonadotrophin secretion and sexual behaviour (Beltramino and Taleisnik 1978, Bagga *et al.* 1984, Chateau *et al.* 1984, Salamon *et al.* 2005). The MeA has been shown to exert an inhibitory effect on the reproductive axis. Indeed, electrical stimulation of the MeA delays puberty onset in female rats (Bar-Sela and Critchlow 1966). Conversely, lesioning of MeA advances puberty onset (Li *et al.* 2015). Furthermore, lesioning of MeA increases circulating LH levels (Lawton and Sawyer 1970) and prevents the stress-induced suppression of pulsatile LH release (Lin *et al.* 2011). Neuroanatomical studies have shown evidence of direct projections from the MeA to the hypothalamic regions that regulate reproductive hormone release such as Kiss1-rich ARC and AVPV, and GnRH-rich mPOA (Hahn and Coen 2006, Usunoff *et al.* 2009, Keshavarzi *et al.* 2014). MeA may exert its inhibitory effect on the reproductive system via these projections. However, the neurochemical phenotypes of these projections are still to be investigated.

Recently, Kiss1-Kiss1r signalling has been identified in the MeA (Lee *et al.* 1999, Clarkson *et al.* 2009). Previous studies regarding the physiological functions of Kiss1 signalling have mainly focused on two regions of the hypothalamus; the ARC and AVPV. Numerous studies have indicated that ARC and AVPV Kiss1 signalling are involved in LH pulse and surge generation, respectively. Knockdown of *Kiss1* in the ARC decreases LH pulse frequency, while knockdown of *Kiss1* in the AVPV reduces the incidence of LH surges in female rats (Beale *et al.* 2014, Hu *et al.* 2015). Knockdown of *Kiss1* in the ARC and AVPV both resulted in a disruption of oestrous cyclicity in female rats (Beale *et al.* 2014, Hu *et al.* 2015). However, Kiss1 signalling is not restricted to hypothalamic regions. Both Kiss1 neurones and fibres are found in extra-hypothalamic regions such as the MeA (Clarkson *et al.* 2009). Furthermore, *Kiss1r* mRNA is also expressed in the MeA (Lee *et al.* 1999). It was shown that the expression of *Kiss1* mRNA in the MeA is regulated by the stages of oestrous cycles of female rats and the levels of sex steroids of both sexes (Kim *et al.* 2011). The expression of *Kiss1* mRNA in the MeA was highest at proestrus and amplified by sex steroids in castrated rats of both sexes (Kim *et al.* 2011). The enhanced Kiss1 signalling in the MeA by E_2 treatment appears to be regulated by ER-dependent pathways, since nonaromatisable androgens do not modulate *Kiss1* expression in the MeA (Kim *et al.* 2011). Indeed, both *ERα* and *ERβ* mRNA expression were detected in the MeA in rats of both sexes (Cao and Patisaul 2013).

In addition to its regulation by gonadal steroids, MeA Kiss1 expression is modulated by GABA neurotransmitter. The majority of hypothalamic and MeA Kiss1 neurones express $GABA_B$ receptor (Di Giorgio *et al.* 2014). In adult mice of both sexes, Kiss1 signalling in the MeA is under tonic inhibitory regulation by GABAergic signalling, since knockout of $GABA_B$ receptor amplified the expression of *Kiss1* mRNA in the MeA and increased the frequency of pulsatile GnRH release (Catalano *et al.* 2010, Di Giorgio *et al.* 2014). This inhibitory effect of $GABA_B$ receptor signalling on Kiss1 signalling was not found in the ARC or the AVPV in adult mice (Di Giorgio *et al.* 2014). Therefore, $GABA_B$ receptor signalling appears to modulate the Kiss1 signalling by a regional specific manner. These studies have heightened interest in the role of amygdala Kiss1 signalling. However, unlike hypothalamic Kiss1 signalling, whether the Kiss1 signalling in the MeA plays a role in regulating reproductive function including ovulation, oestrous cyclicity, or gonadotrophin secretion is still unknown.

The interactions between Kiss1 and GABAergic signalling systems appear to be reciprocal. Various studies have indicated that Kiss1 signalling can modulate central GABAergic signalling with different effects. Kiss1 can increase GABAergic transmission directly to GnRH neurones and also potentiate the postsynaptic response of GnRH neurones to GABA (Pielecka-Fortuna and Moenter 2010, Ronnekleiv and Kelly 2013). However, other studies have showed Kiss1 can decrease the release of GABA in the mPOA (Neal-Perry *et al.*

2009) and block $GABA_B$ receptor mediated inhibitory effect on GnRH neuronal activity (Zhang *et al.* 2009). Furthermore, recent studies have suggested that Kiss1 might possibly modulate GABAergic signalling in the MeA. Peripheral administration of Kiss1 increased LH secretion concomitant with reduced neuronal activity in the amygdala measured by magnetic resonance imaging (MRI) (Comninos *et al.* 2015). GABA is the chief inhibitory neurotransmitter in the mammalian central nervous system. GABAergic neurones form an interconnected local inhibitory network in the MeA and constitute the majority of projections out from this region (Keshavarzi *et al.* 2014). Therefore, peripheral administered Kiss1 might affect the GABAergic signalling in the MeA to induce the LH release.

7.2 Aims and objectives

1. To test the hypothesis that Kiss1 signalling in the MeA regulates both the pulsatile and surge modes of LH release in female rats.

a) To determine the effect of intra-MeA administered Kiss1r agonist and antagonist on pulsatile LH secretion in OVX rats.

b) To assess the effect of chronic administered Kiss1r antagonist on spontaneous LH surges and oestrous cyclicity in intact female rats.

2. To test the hypothesis that the stimulatory effects of peripherally administered Kiss1r agonist on LH secretion is mediated via the $GABA_B$ receptor in the MeA in OVX rats.

7.3 Materials and methods

7.3.1 Animals and surgical procedures

Adult Sprague-Dawley rats were OVX and implanted with bilateral guide cannulae directed towards the MeA (see section 2.2.2.5). An additional group of rats used as controls were OVX and implanted with unilateral icv guide cannulae directed towards the left lateral cerebral ventricle (see section 2.2.2.4). After a 10-d recovery period (Fig. 7.1, A), rats were fitted with two indwelling cardiac catheters via the jugular vein, as described in section 2.2.2.6. Experimentation commenced 3 d later (Fig. 7.1, A).

A separate group of adult female rats were chronically implanted bilaterally with cannulae (28 gauge, Plastics One) directed towards the MeA: coordinates for implantation were 3.4 mm lateral, 3.14 mm posterior to bregma, and 8.6 mm below the surface of the dura according to the Rat Brain Atlas of Paxinos and Watson (Paxinos G 1986). An osmotic mini-pump (Model 1002, Alza Corp) was attached to the left cannula with silicone tubing, and implanted subcutaneously in the interscapular space. This was repeated for the right cannulae. The Kiss1r antagonist peptide-234 (Sigma-Aldrich; n = 10) or aCSF (n = 7) was delivered

bilaterally via the osmotic mini-pump at the rate of 0.25 µl/h for a 14-d period. The dose of peptide-234 used was 0.33 nmol/µl (2 nmol/d for each side). An additional group of female rats used as controls were implanted a cannula (28 gauge, Plastics One) directed towards the left lateral cerebral ventricle: the coordinates for implantation were 1.5 mm lateral, 0.6 mm posterior to bregma, and 4 mm below the surface of the dura according to the Rat Brain Atlas of Paxinos and Watson (Paxinos G 1986). An osmotic mini-pump (Model 2002, Alza Corp) was attached to the cannula. To compare with bilaterally 2 nmol/d intra-MeA administration of peptide-234, peptide-234 (4 nmol/d) was administered via the mini-pump to the ventricle system. After a 3-d recovery period (Fig. 7.1, B), rats were fitted with two indwelling cardiac catheters via the jugular vein, as described in section 2.2.2.6. Experimentation commenced 2 d later (Fig. 7.1, B).

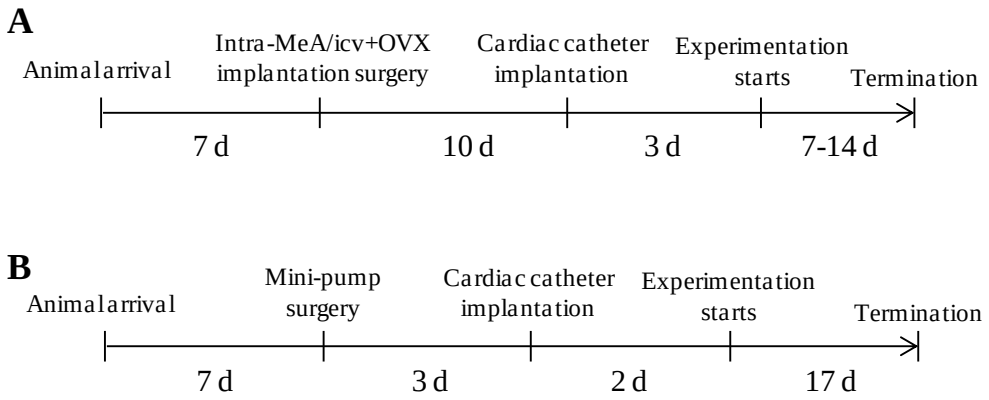

Fig. 7.1 Timeline diagram of various surgical and experimental procedures for investigating the role of MeA Kiss1 signalling in regulating the release of LH and the role of MeA $GABA_B$ receptor signalling in mediating the release of LH induced by Kiss1.

7.3.2 Experiment 1: Effects of intra-MeA administration of Kiss1r agonist or antagonist on LH release in OVX rats

On the morning of experimentation, a bilateral internal injection cannula (Plastics One) with extension tubing preloaded with aCSF, Kp-10 (Sigma-Aldrich) or peptide-234 was inserted into the guide cannula and extended 1 mm beyond the guide cannula to reach the MeA. The distal end of the tubing was extended outside of the cage connected to a 5 µl Hamilton syringe (Waters International), thereby allowing remote infusion without disturbing the rat during the experiment (see section 2.2.3.3). Rats were also attached via one of the two cardiac catheters to the computer-controlled automated blood sampling system (see section 2.2.3.1). Once connected, animals

were left undisturbed for 1 h. Blood sampling commenced between 1000 and 1100 h and samples were collected every 5 min for 6 h for LH measurement.

Kp-10 or vehicle (aCSF) was infused (intra-MeA) over 5 min into OVX rats after 2 h of controlled blood sampling. Rats received a single dose of 100 pmol or 1 nmol Kp-10 in 400 nl aCSF (n = 9 per treatment group), bilaterally. Control rats received 400 nl aCSF (n = 7). Peptide-234 (50 pmol) was administered in 400 nl aCSF over 5 min after 2 h of controlled blood sampling and then the same dosage was repeated on two further occasions at an interval of 30 min (n = 9). The above dosage was selected based on our previous studies investigating the Kiss1 signalling in the ARC (Li *et al.* 2009b).

Additional group of OVX rats implanted with icv cannulae were used as controls to rule out the possibility that Kp-10 administered into the MeA may have diffused into the lateral cerebral ventricle to exert its effect via that route. Rats were attached to the automated blood sampling system and equipped with the icv injection system on the morning of experimentation, as described in Chapter 4. Kp-10 (200 pmol in 4 µl aCSF) was infused via the icv cannulae over 5 min into OVX rats (n = 6) after 2 h of controlled blood sampling. This dose was selected in order to compare with bilaterally intra-MeA administered Kp-10 (100 pmol).

7.3.3 Experiment 2: Effects of chronic intra-MeA administration of Kiss1r antagonist on oestrous cyclicity and the proestrus LH surges in intact female rats

After the implantation of the osmotic mini-pump filled with peptide-234 or aCSF (see section 7.3.1), rats (17 rats from the experiment of chronic intra-MeA administration of peptide-234/aCSF via mini-pump and 5 rats from the experiment of chronic icv administration of peptide-234/aCSF via mini-pump) were monitored daily (0900-1000 h) for normal ovarian cyclicity by means of vaginal cytology for 22 d. Normal ovarian cyclicity in this study was defined as having at least 2 consecutive normal cycles, which last for 4-5 d with 1-2 d of oestrus (Hu *et al.* 2015).

At least 2 d after the implantation of cardiac catheters (see section 7.3.1), rats that were shown to be in proestrus were attached via one of the two cardiac catheters to the computer-controlled automated blood sampling system (see section 2.2.3.1). Blood sampling commenced at 1300 h and samples (25 µl) were collected every 30 min for 7 h for LH measurement.

7.3.4 Experiment 3: Effects of peripheral administration of Kiss1r agonist on LH release in the presence of intra-MeA administration of $GABA_B$ receptor antagonist in OVX rats

On the morning of experimentation, OVX rats were attached to the automated

blood sampling system and equipped with the intra-MeA injection system, as described above. After 1 h 45 min of controlled blood sampling, rats received three consecutive injections (intra-MeA) of $GABA_B$ antagonist CGP35348 (1.2 nmol in 400 nl aCSF, n = 6) or vehicle (400 nl aCSF, n = 6), each administered over 5 min, 30 min apart, with a further injection (iv) of Kp-10 (50 nmol in 0.2 ml saline), administered 15 min following the first injection of CGP35348 or aCSF. Additional rats, used as controls, received three consecutive injections (intra-MeA, 30 min apart) of CGP35348 (1.2 nmol, n = 4) after 1 h 45 min of controlled blood sampling with a further injection of saline (0.2 ml) after the first injection of CGP35348. The dose of CGP35348 was based on our previous studies investigating GABAergic signalling in the mPOA (Lin *et al.* 2012).

7.3.5 Statistical analysis

Detection of LH pulses in OVX rats in experiment 1 was established by use of the algorithm ULTRA (Van Cauter 1988). The effects of pharmacological agents on pulsatile LH secretion were analysed by comparing the mean LH pulse interval in the 2-h period preceding treatment with that in the 1-h post-treatment and 2-4-h post-treatment period. The duration (min) of the 2-h pre-treatment, 1-h post-treatment and 2-4 h post-treatment period, was divided by the number of LH pulses detected in each of these periods to give the appropriate LH pulse interval. The effects of pharmacological agents on overall LH release in

experiment 1 were calculated by comparing the average AUC per h in the 1-h and 2-4-h post-treatment periods with that in the 2-h pre-treatment period, using SigmaPlot version 11 (Systat Software). In experiment 2, the percentage of rats exhibiting LH surges/normal oestrus cyclicity were calculated by the number of rats showing LH surges/normal oestrus cycles divided by the number of overall rats. In experiment 3, the effects of pharmacological agents on overall LH release were calculated by comparing the AUC in 1st and 2nd 2-h post-treatment period with that in the 2-h pre-treatment period. Statistical significance for the percentage of normal oestrous cyclicity and the percentage of rats exhibiting LH surges was examined by Fisher's test. All other results were analysed by one-way ANOVA followed by Dunnett's test. All data were shown as mean ± S.E.M. $P < 0.05$ was considered statistically significant.

7.4 Results

7.4.1 Experiment 1: Effects of intra-MeA administration of Kiss1r agonist or antagonist on LH release in OVX rats

To investigate the role of Kiss1 signalling in the MeA in pulsatile LH release, we examined the effects of intra-MeA administered Kp-10 or its antagonist, peptide-234, on LH secretion in OVX rats. Intra-MeA administration of Kp-10 induced a dose-dependent increase in overall LH levels calculated by AUC (Figs. 7.2, B, C and F). 100 pmol Kp-10 increased the overall LH levels per h by 17.2%

during the 1-h post-infusion period (AUC per h, 2-h pre-injection *vs*. 1-h post-injection: 286.7 ± 3.8 *vs*. 336.0 ± 13.2 ng/ml.min; $P < 0.05$) and by 69.5% with 1 nmol Kp-10 (AUC per h, 2-h pre-injection *vs*. 1-h post-injection: 286.4 ± 11.0 *vs*. 485.5 ± 37.9 ng/ml.min; $P < 0.05$). By contrast, intra-MeA administration of peptide-234 inhibited the overall LH levels during the 2-4-h post-infusion period (AUC per h, 2-h pre-injection *vs*. 2-4-h post-injection: 290 ± 18.8 *vs*. 209.7 ± 17.5 ng/ml.min; $P < 0.05$; Figs. 7.2, D and F). Furthermore, at the dose of 100 pmol, Kp-10 did not affect the LH pulse frequency (Figs. 7.2, B and E). However, 1 nmol Kp-10 induced a profound and prolonged increase in LH secretion that precluded reliable LH pulse detection in this treatment group. By contrast, intra-MeA administration of peptide-234 inhibited the LH pulse frequency in OVX rats (LH pulse interval, 2-h pre-injection *vs.* 2-4-h post-injection: 22.0 ± 0.8 *vs*. 55.3 ± 5.6 min; $P < 0.05$; Figs. 7.2, D and E). In the control group, vehicle (aCSF) did not affect the pulsatile LH release or overall LH secretion (Figs. 7.2, A, E and F). In addition, the MeA cannulae were found to be misplaced in one rat (Fig. 7.5) in which both Kp-10 and peptide-234 did not affect the LH secretion. Furthermore, icv administration of Kp-10 (200 pmol) did not affect the LH secretion in OVX rats (AUC per h, 2-h pre-injection *vs*. 1-h post-injection: 287.6 ± 7.7 *vs*. 309.4 ± 13.7 ng/ml.min; $P > 0.05$).

7.4.2 Experiment 2: Effects of chronic intra-MeA administration of Kiss1r antagonist on oestrous cyclicity and the proestrus LH surges in intact female rats

To investigate the role of Kiss1 signalling in the MeA on the surge mode of LH secretion, we examined the effects of intra-MeA administered Kiss1r antagonist peptide-234 on oestrous cyclicity and LH secretion in intact female rats. Chronic intra-MeA administration of peptide-234 (2 nmol/d bilaterally) reduced the incidence of normal oestrous cyclicity in female rats by 61.4% (aCSF *vs*. peptide-234, 71.4% vs. 10.0%, $P < 0.05$; Figs. 7.3, A and B). Furthermore, peptide-234 reduced the percentage of rats showing spontaneous LH surges by 65.7% (aCSF *vs*. peptide-234, 85.7% vs. 20%, $P < 0.05$; Fig. 7.3, E). Only 1 out of 10 animals exhibited an LH surge at proestrus in the peptide-234 treatment group, whereas 6 out of 7 showed LH surges in the aCSF control group. Representative examples of LH surges in control and treatment groups are provided in Figures 7.2, C and D. No misplaced cannulae were found in this set of experiments (Fig. 7.5). Furthermore, in additional rats chronically administered (icv via a mini-pump) peptide-234 (4 nmol/d), 4 out of 5 exhibited spontaneous LH surges in proestrus stage, and 3 out of 5 showed normal oestrus cycles.

7.4.3 Experiment 3: Effects of peripheral administration of Kiss1r agonist on LH release in the presence of intra-MeA administration of $GABA_B$ receptor antagonist in OVX rats

To investigate the role of $GABA_B$ receptor signalling in the MeA in Kiss1-induced LH secretion, we examined the effects of iv administered Kp-10 in the presence of intra-MeA administration of the $GABA_B$ receptor antagonist CGP35348 on LH secretion in OVX rats. Intra-MeA administration of CGP35348 attenuated the rise in overall LH secretion induced by iv Kp-10 (Fig. 7.4). 50 nmol Kp-10 (iv) induced a robust LH secretion (calculated by AUC) during both 1^{st} and 2^{nd} h post-administration periods ($P < 0.05$). This increase was significantly attenuated by co-administration of CGP35348 during the 2^{nd} 2-h post-administration period (AUC in 2^{nd} 2-h post-injection, vehicle + Kp-10 *vs*. CGP35348 + Kp-10: 786.4 ± 31.2 *vs*. 680.1 ± 32.2 ng/ml.min, $P < 0.05$; Figs. 7.4, B, C and D). CGP35348 *per se* did not affect the overall LH secretion (Figs. 7.4, A and D). In addition, the MeA cannulae were found to be misplaced in one rat (Fig. 7.5). In that rat, CGP35348 did not seem to affect the LH secretion stimulated by iv Kp-10.

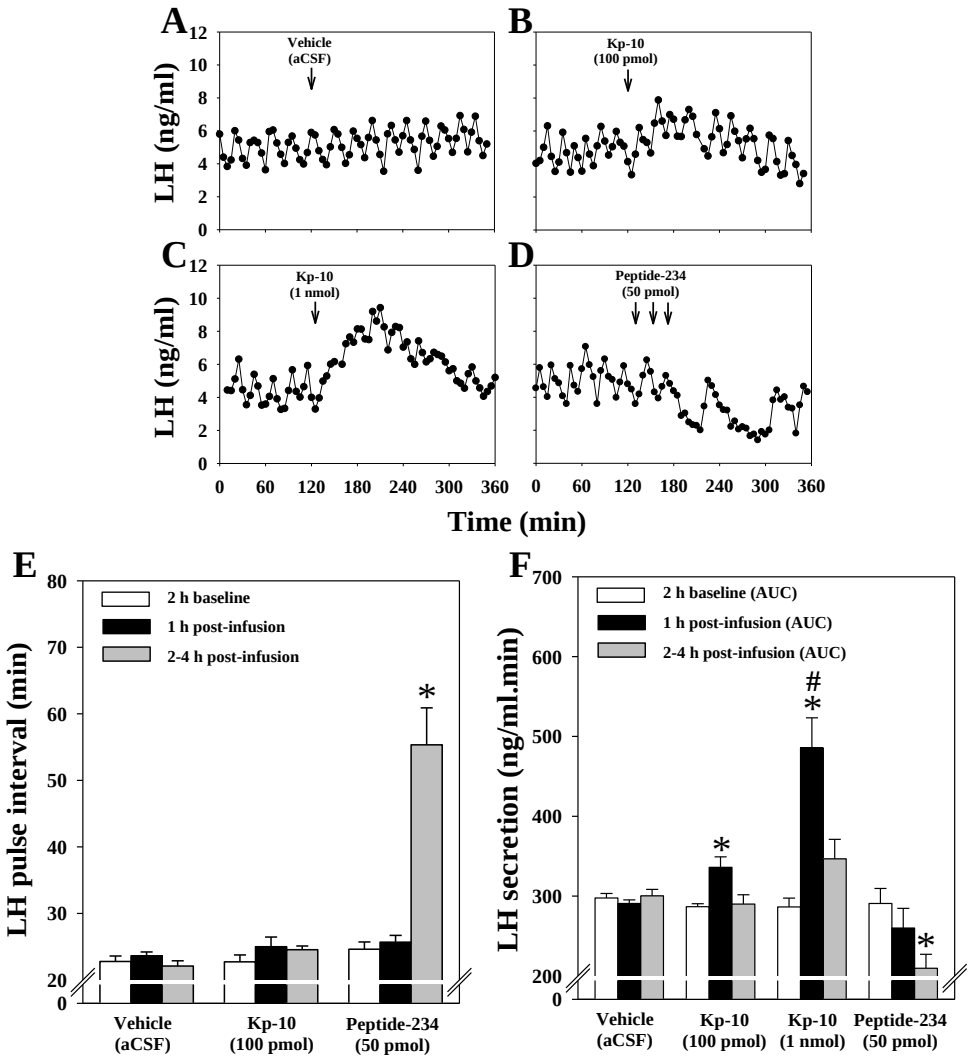

Fig. 7.2 Effects of intra-MeA administration of Kiss1r agonist or antagonist on LH release in OVX rats. Representative LH profiles illustrating effects of intra-MeA administration (arrow) of Kp-10 (B-C), peptide-234 (D) or vehicle (aCSF) (A) in OVX rats. Kp-10 (100 pmol) had no significant effects on pulsatile LH secretion in OVX rats. However, peptide-234 (3×50 pmol) inhibited the pulsatile LH release, as summarised in panel E. Furthermore, Kp-10 induced a dose-dependent increase of LH secretion, calculated by AUC, as summarised in panel F. By contrast, peptide-234 inhibited the LH secretion. *, $P < 0.05$ *vs.* 2-h baseline period within the same group, as well as the same 2-h period within the vehicle treated group; #, $P < 0.05$ *vs.* same 2-h period within the group treated with 100 pmol Kiss1 (n = 7-9 per group).

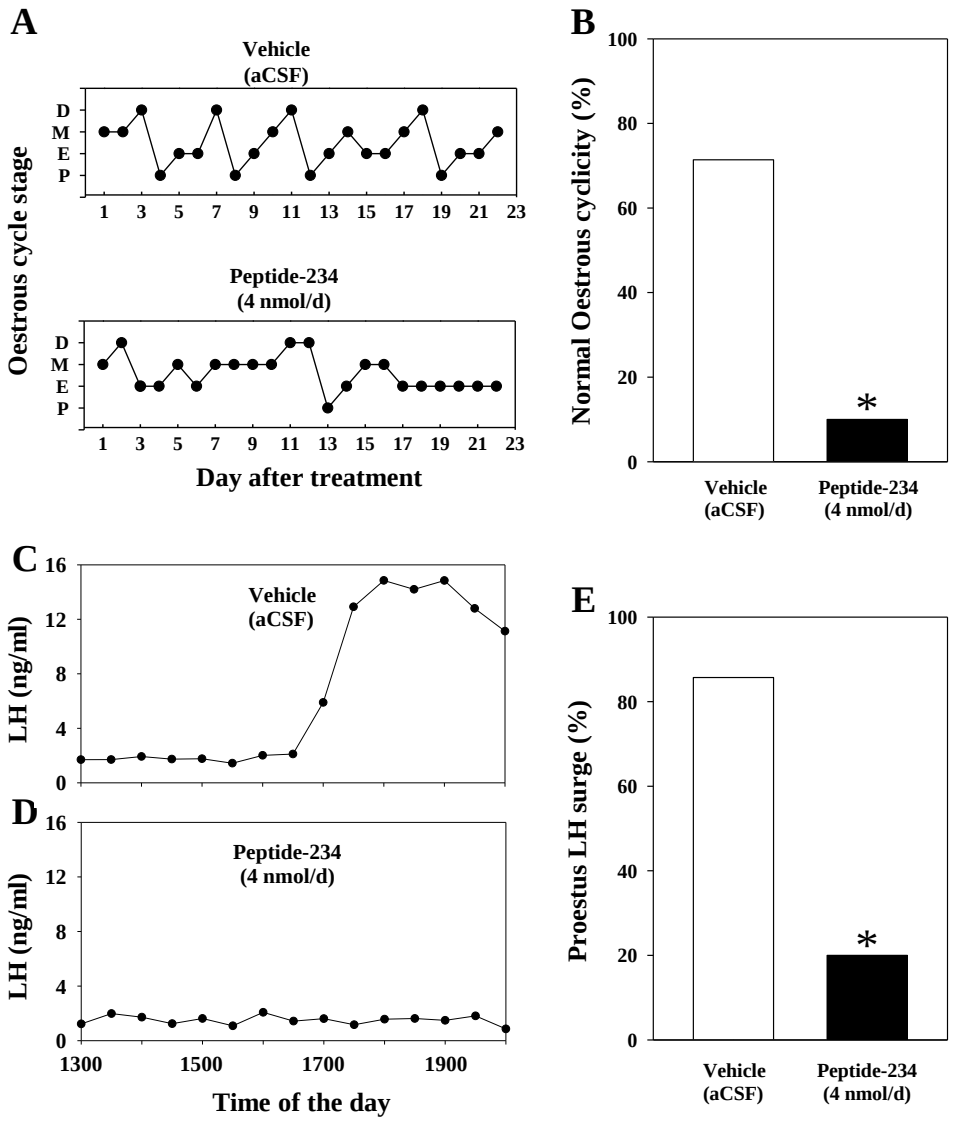

Fig. 7.3 Effects of chronic intra-MeA administration of Kiss1r antagonist on oestrous cyclicity and proestrus LH surge in intact female rats. (A) Representative oestrous cycle profiles of rats administered (intra-MeA, bilaterally) vehicle (aCSF, 4 µl/d) or peptide-234 (2 nmol/d); P = proestrus, E = oestrus, M = metoestrus, D = dioestrus. Peptide-234 reduced the percentage of rats showing normal oestrous cyclicity, as summarised in panel B. (C, D) Representative LH surge profiles in rats administered (intra-MeA, bilaterally) aCSF or peptide (2 nmol/d), respectively. The percentage of rats exhibiting spontaneous LH surges was reduced by peptide-234, as summarised in panel E. *, $P < 0.05$ *vs.* vehicle treated group (n = 7-10 per group).

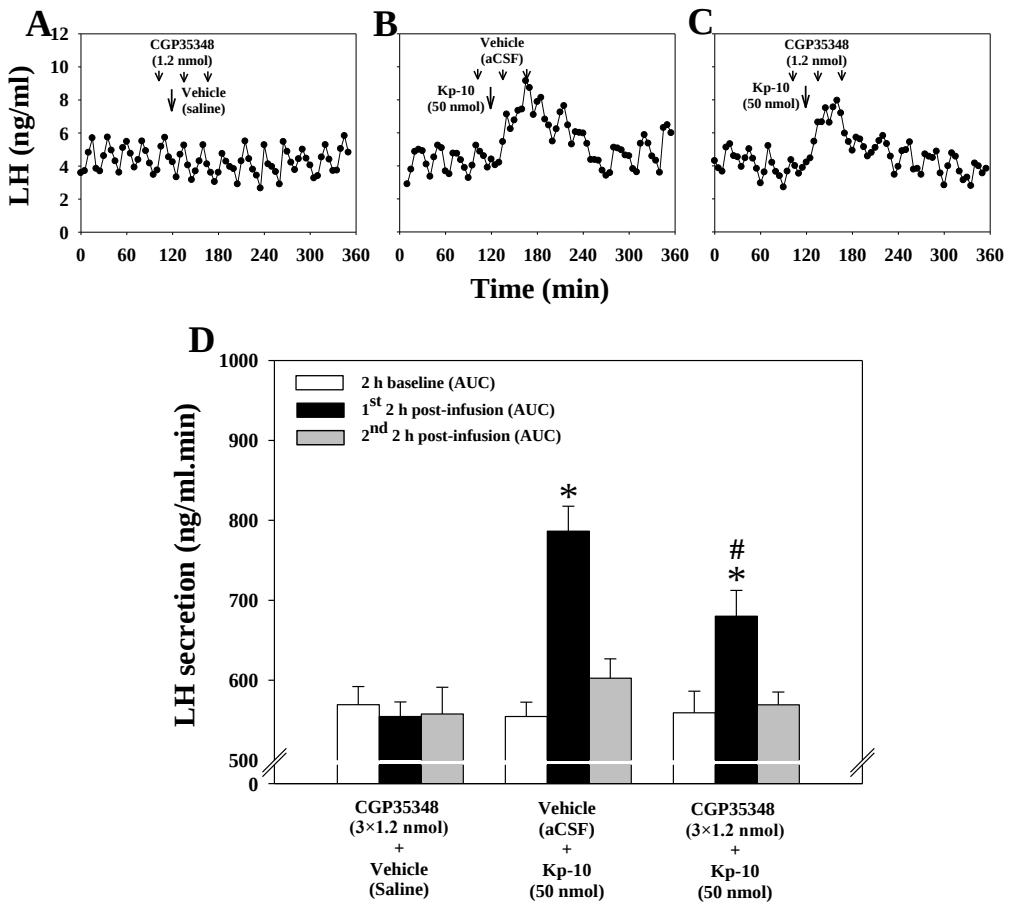

Fig. 7.4 Effects of peripheral administration of Kiss1r agonist on LH release in the presence of intra-MeA administration of $GABA_B$ antagonist in OVX rats. Representative LH profiles illustrating the effect of iv administration of 50 nmol Kp-10 (arrow) in the presence (C) or absence (B) of intra-MeA administration of 3×1.2 nmol CGP35348 (↓↓↓), as well as that of 3×1.2 nmol CGP35348 (A) (↓↓↓) alone, in OVX rats. The Kp-10-induced LH secretion was blocked by pre-treatment of CGP35348, as summarised in panel D. *, $P < 0.05$ *vs.* 2-h baseline period within the same group, as well as the same 2-h period within the vehicle treated group; #, $P < 0.05$ *vs.* same 2-h period within the group treated with vehicle + Kp-10 (n = 4-6 per group).

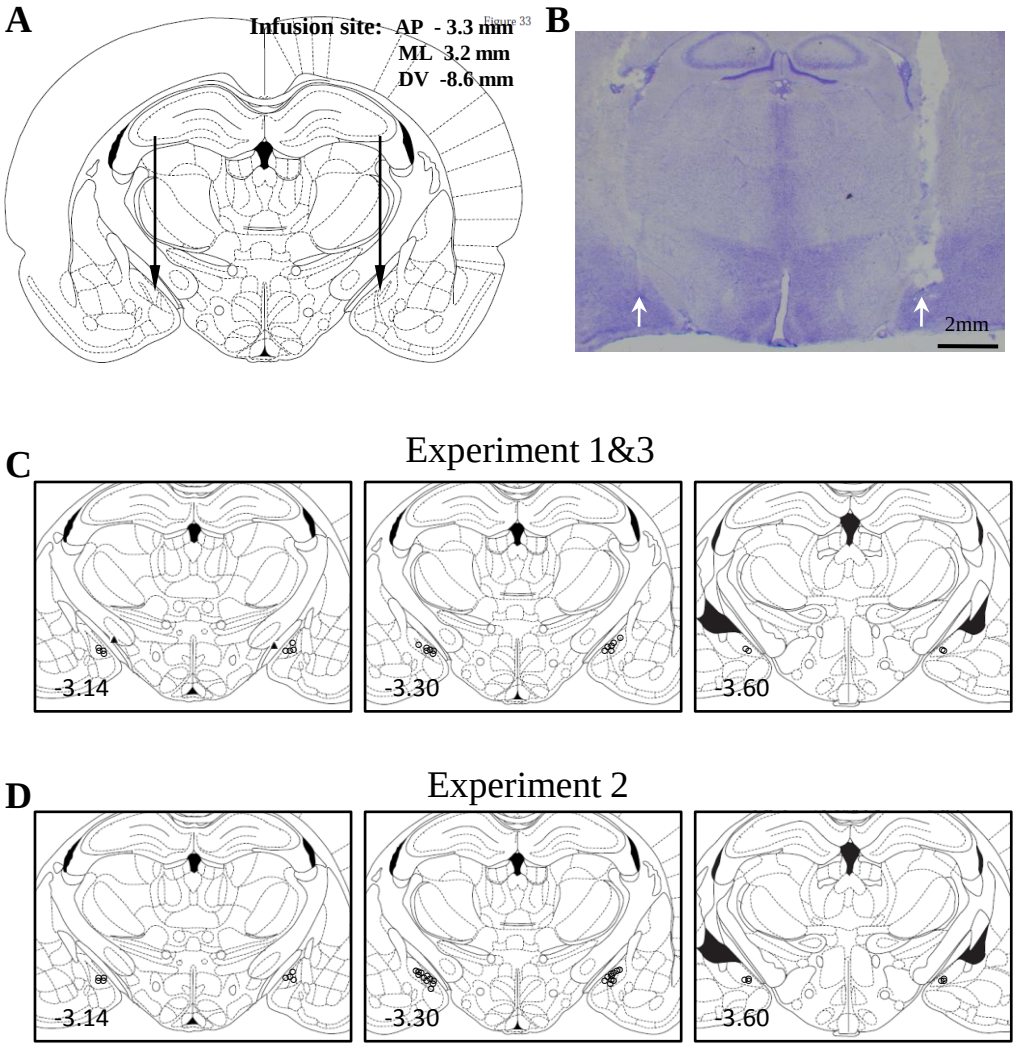

Fig. 7.5 Schematic illustration and photomicrograph of the infusion site targeted to the MeA. A, schematic illustration showing the target site for bilateral cannulation of the MeA. Arrows point to the location of the tips of internal cannulae or 28 gauge cannulae. B, photomicrograph of a coronal brain section in a representative animal implanted with a bilateral cannula in the MeA. Arrows indicate the tips of the internal cannulae. C-D, schematic drawings of the MeA illustrating the individual sites of injection for the experiment 1&3 and 2, respectively. Numbers in each drawing indicate the distance (mm) to Bregma. Open circles show the right injection sites. Close triangles show the misplaced injection sites.

7.5 Discussion

The present study investigated whether Kiss1 signalling in the MeA affects LH secretion. We found that intra-MeA administration of Kiss1r agonist, Kp-10, caused a dose-dependent increase of LH secretion in OVX rats. Previous studies have shown that intra-mPOA and intra-ARC administered Kp-10 induce a robust LH secretion (Li *et al.* 2009b). A lower dose of Kp-10 was required in the mPOA and ARC to induce a comparable LH secretory response compared with the MeA (Li *et al.* 2009b). This may be due to a less robust expression of *Kiss1r* mRNA in the MeA compared with the mPOA or ARC (Lee *et al.* 1999). In order to rule out the possibility that Kp-10 injected into the MeA may have leaked from this site into the ventricular system to have an affect on Kiss1r in the ARC or mPOA, we injected the same dose of Kp-10 into the ventricular system. It was shown that icv administration of 200 pmol Kp-10 did not affect LH secretion in OVX rats. This is also in agreement with previous studies showing that icv administration of 100 or 300 pmol Kp-10 did not stimulate LH secretion (Thompson *et al.* 2004, Pheng *et al.* 2009). This is a critical observation since it strongly indicates that the Kp-10 administered into the MeA exerted its effect locally and not via leakage into the ventricular system to target hypothalamic sites.

The present study has also shown that intra-MeA administration of Kiss1r antagonist peptide-234 profoundly suppresses LH pulse frequency. Again, icv administration of the same dose of peptide-234 did not affect the LH secretion,

indicating a localised site of action to the amygdala. These data suggest that Kiss1-Kiss1r signalling in the MeA can regulate the GnRH pulse generator frequency. Previous studies have implicated MeA in the regulation of the GnRH pulse generator. Lesioning of the MeA did not affect basal pulsatile LH release, whereas it blocked psychological stress-induced suppression of LH pulse frequency (Lin *et al.* 2011). It is therefore possible that the MeA may be sensitive to psychological stimuli and function as an upstream 'inhibitory brake' on the hypothalamic GnRH pulse generator. The psychological stress-induced suppression of LH pulses was associated with a decrease in *Kiss1* mRNA expression in the mPOA and ARC (Kinsey-Jones *et al.* 2009). Given the inhibitory effect of Kiss1r antagonism in the MeA on the pulsatile LH release, it is possible that Kiss1 signalling in the MeA is involved in stress-induced suppression of pulsatile LH release. However, the neuroanatomical circuitry underlying this remains to be elucidated. It has been shown that the majority of projections from the MeA to the hypothalamus are GABAergic (Keshavarzi *et al.* 2014), which is in keeping with inhibitory role of MeA on the reproductive axis. Numerous studies have indicated that GABA signalling plays an important inhibitory role in the regulation of GnRH release. Local infusion of GABA into the mPOA or ARC suppressed the pulsatile LH release (Jarry *et al.* 1991, Ferreira *et al.* 1996). Therefore, Kiss1-Kiss1r signalling in the MeA may affect the extrinsic projections of GABAergic signalling to affect the pulsatile LH release. Indeed, it has been shown that Kiss1 signalling can affect the

GABAergic signalling since direct injections of Kiss1 inhibit GABA release in the mPOA (Neal-Perry *et al.* 2009). More studies will be needed to investigate the potential effect of the MeA Kiss1 signalling on the GABAergic signalling. However, it should be noted that the present study has also shown that intra-MeA administration of Kp-10 (100 pmol) did not affect the pulsatile LH release. Unfortunately, we cannot analyse the LH pulsatility following the injection of higher dosage of Kp-10 due to the dramatic increase of LH levels. This dramatic increase of LH release may be due to a massive increase of LH pulse frequency. However, a caveat may exist since previous studies have shown that iv administered Kp-10 did not affect the frequency of GnRH pulse generator (reflected by the frequency of MUA volleys) in OVX rats, despite its dramatic stimulatory effects on the LH secretion (Kinsey-Jones *et al.* 2008). Taken together, Kiss1 signalling in the MeA appears to be an important regulator of the GnRH pulse generator and warrants further investigation.

Kiss1 signalling in the MeA fluctuates during the oestrous cycle, with high expression of *Kiss1* mRNA at proestrus stage, when circulating levels of E_2 are highest (Kim *et al.* 2011). ERs are highly expressed in the MeA (Simerly *et al.* 1990, Greco *et al.* 2001). Treatment with E_2 dramatically increased the expression of *Kiss1* mRNA in the MeA of OVX rats (Kim *et al.* 2011). This indicates that E_2 positively regulates Kiss1 signalling in the MeA, which mirrors that of Kiss1 signalling in the AVPV. Therefore, we ventured to investigate the role of Kiss1 signalling in the MeA on spontaneous LH surges generation and

oestrous cyclicity in rats. The present study has shown that intra-MeA administration of Kiss1r antagonist reduced the percentage of animals showing spontaneous LH surges and normal oestrous cyclicity. In contrast, the majority of rats administered the same dose of Kiss1r antagonist via an icv route showed normal spontaneous LH surges and oestrous cyclicity. This suggests that the effect of intra-MeA administered Kiss1r antagonist on the LH surges and oestrous cyclicity is unlikely to be due to leakage into the ventricular system. The effect of Kiss1r antagonism in the MeA is in line with a recent study showing that knockdown of Kiss1 signalling in the AVPV of rats resulted in abnormal oestrous cyclicity and a reduction in the occurrence of spontaneous LH surges (Hu *et al.* 2015). The disrupted oestrous cyclicity was characterised by an increased time spent in oestrus and metoestrus stages of the cycle (Hu *et al.* 2015), which is in line with the present study. Similarly, Kiss1, Kiss1r or Kiss1 ERα knockout mice move between oestrus and dioestrus stages but remain anovulatory (Chan *et al.* 2009, Mayer *et al.* 2010). This may suggest that animals get stuck in the preovulatory phases as the E_2-Kiss1-GnRH signalling cascade essential for the LH surge and as a result ovulation is inhibited. Furthermore, lesioning of the MeA blocked ovulation (Bagga *et al.* 1984) and disrupted normal oestrous cyclicity (Li *et al.* 2015) in rats. Taken together with the present study, Kiss1 signalling in the MeA may be key component in the signalling pathways that initiate spontaneous LH surges.

The present study also investigated the role of $GABA_B$ receptor signalling in the MeA in Kiss1-induced LH secretion. Injection of $GABA_B$ receptor antagonist into the MeA attenuated peripherally administered Kp-10-induced LH secretion. This suggests that Kp-10-induced LH secretion is partially dependent on the $GABA_B$ receptor signalling in the MeA. We can not rule out the possibility that Kp-10 may also require $GABA_A$ receptor to induce LH secretion. Recent studies have shown that increased LH secretion in response to peripherally administered Kp-10 was associated with a marked reduction of neuronal activity in the MeA measured by MRI (Comninos *et al.* 2015). It is possible that this reduction could represent an inhibition of the GABAergic neuronal activities in the MeA, since most neurones in the MeA are GABAergic (Mugnaini and Oertel 1985, Keshavarzi *et al.* 2014). Furthermore, the majority of projections out of the MeA to reproductive related hypothalamus nucleus are GABAergic (Keshavarzi *et al.* 2014). Therefore, this inhibited GABAergic signalling is speculated to eventually lead to the activation of GnRH neurones and resultant LH release via a disinhibition of inhibitory effect. It is important to consider that MRI measurement cannot distinguish directly between activation of one population of neurones and deactivation of another, and hence represents an overall net effect. Additionally, peripherally administered Kp-10 is not thought to cross the blood-brain barrier (Herde *et al.* 2011). It has been reported that iv administration of Kp-10 induced neuronal activity in the supraoptic nucleus to release oxytocin (Scott and Brown 2011). The mechanism for this modulation is

unknown, but could possibly be due to the activation of an afferent vagal pathway (Scott and Brown 2011). Similarly, the inhibited neuronal activities in the MeA in response to peripherally administered Kp-10 (Comninos *et al.* 2015) may be via an indirect route, which remains to be elucidated.

In summary, the present study has shown that Kiss1 signalling in the MeA is involved in both LH pulse and LH surge generation. Our data provide evidence that Kiss1 in the MeA is also an essential regulator of the reproductive system, integrating limbic circuits with the modulation of the HPG axis. Furthermore, Kiss1-induced LH secretion is partially dependent on $GABA_B$ signalling in the MeA, which indicates an indirect pathway may exist for Kiss1 signalling to act on the GnRH neurones.

CHAPTER EIGHT: GENERAL DISCUSSION

Sexual maturation involves a series of neuroendocrine developments that lead to the activation of the HPG axis. This activation is initiated by an acceleration of the GnRH pulse generator, which results in the accelerated pulsatile release of GnRH, the principle central stimulator of gonadotrophins. This phenomenon has been found across mammalian species, from rodents to primates and including humans (Watanabe and Terasawa 1989, Sisk *et al.* 2001, Harris and Levine 2003, McCartney 2010, Li *et al.* 2012). The timing of sexual maturation appears to be tightly regulated by the activity of the GnRH pulse generator. Recent studies have implicated Kiss1 and NKB signalling in the process of sexual maturation. It has been shown that humans with inactivating mutations in genes encoding KiSS1, KiSS1r, NKB or NK3R lack pubertal development (de Roux *et al.* 2003, Seminara *et al.* 2003, Topaloglu *et al.* 2009, Topaloglu *et al.* 2012).

The first aim of this project was to examine the role of NKB-NK3R signalling in regulating the timing of puberty onset and the dynamic release of LH in the prepubertal stage of rats. It has been demonstrated in the present study that chronic central administration of NK3R antagonist results in a significant delay in the timing of puberty onset, which is in keeping with the phenotype of human genetic mutations (see section 3.3.2). Interestingly, it has been shown that *NK3R*-null mice display normal timing of puberty onset, although with disrupted oestrous cycles and reduced gonadal weight (Yang *et al.* 2012). Similarly, the pharmacological tools using NK3R agonists or antagonists have yielded

conflicting results in rodent studies (Gill *et al.* 2012, Navarro *et al.* 2012, Nakahara *et al.* 2013). This could be due to differences in the dosage or the routes of administration. Furthermore, it has been shown in the present study that NK3R antagonism reduced the accelerated increase of pulsatile LH release in prepubertal rats, which may underlie the delay in puberty onset (see section 3.3.3). It has previously been shown that chronic infusion of NK3R agonist advances puberty onset, but only with a tendency to increase pulsatile LH release in female rats (Nakahara *et al.* 2013). Taken together, NKB-NK3R signalling might regulate the timing of sexual maturation by modulating the activity of GnRH pulse generator. Furthermore, blockade of the Dyn receptor resulted in advanced puberty onset, which is associated with increased pulsatile LH release (Nakahara *et al.* 2013). These studies further strengthened the speculation that the oscillation of NKB/Dyn in the ARC produces pulsatile mode of GnRH release, as well as the notion that the GnRH pulse generator regulating the timing of sexual maturation is partially comprised of KNDy neurones. KNDy neurones in the ARC form an interconnected network (Krajewski *et al.* 2010) and ARC NKB/Kiss1 neurones express NK3R (Burke *et al.* 2006, Navarro *et al.* 2009a). It has been speculated that NKB could exert stimulatory effects on KNDy neurones via NK3R to evoke synchronised bursting activities among KNDy neurons, whereas Dyn could extinguish these stimulatory effects (Okamura *et al.* 2013).

The timing of sexual maturation is readily affected by nutritional status. Positive

energy balance leads to precocious puberty onset, while negative energy balance delays puberty. These shifts also appear to be in accordance with the activity of the GnRH pulse generator. Food restriction delays puberty onset (Cheung *et al.* 1997, Navarro *et al.* 2012) and inhibits the pulsatile LH release (Bergendahl *et al.* 1998, Nagatani *et al.* 2000, Forbes *et al.* 2009). In contrast, feeding with a high fat diet induces precocious puberty which is accompanied by an earlier onset of increased frequency of pulsatile LH release (Li *et al.* 2012). It has been shown in the present study that NK3R antagonism delayed the over-nutrition-induced precocious puberty in rats (see section 3.3.2). This delay is associated with a decreased frequency of pulsatile LH release (see section 3.3.4). This indicates that NK3R signalling might regulate the timing of sexual maturation in states of over-nutrition by increasing the activity of GnRH pulse generator. This speculation fits the model that oscillation of NKB/Dyn signalling in the ARC produces pulsatile mode of GnRH release. Modulating this oscillation via NK3R signalling alters the pulsatile mode of GnRH release and therefore shifts the timing of sexual maturation in different nutritional status. This is indirectly supported by previous studies showing that food deprivation delayed the puberty onset in rats which is associated with decreased expression of ARC *Tac2* and *Tacr3* mRNA (Navarro *et al.* 2012). Treatment of NK3R agonists during food restriction could restore the normal timing of puberty onset (Navarro *et al.* 2012). Although little is known about the role of Dyn in regulating puberty onset in different nutritional states, previous studies have established that Dyn plays a

role in integrating nutrition and reproduction (Daniel *et al.* 2013). It was shown that icv administration of Dyn increased food intake in sheep (Baile *et al.* 1987, Della-Fera *et al.* 1990). If each NKB/Dyn oscillation in the ARC could result in a pulse of Kiss1 release, altering the Kiss1 signalling system may affect the timing of sexual maturation in conditions of different nutritional status. It has been shown that food deprivation delayed puberty onset in rats with decreased expression of hypothalamic *Kiss1* mRNA (Castellano *et al.* 2005). This delay could be reversed by treatment of Kiss1r agonists (Castellano *et al.* 2005). Taken together, it can be speculated that the central mechanisms underlying the shifts of timing of sexual maturation in nutritional status might ultimately relay on the ARC KNDy signalling systems which could accelerate the pulsatile GnRH release to initiate puberty. It is certain that more studies will be needed to support this conclusion.

In order to further study the mechanisms underlying the effects of NK3R agonism on LH release, we used pharmacological tools of administering NK3R agonists into conscious adult female rats and assessed the dynamic changes in pulsatile LH release. Central administration (icv) of NK3R agonists induced a triphasic effects on LH release dependent on the oestradiol steroid milieu of the rats (see Chapter 4). More specifically, NK3R agonism induced an inhibitory effect on LH release in OVX rats with no replacement (circulating E_2 levels: 12.7 ± 1.5 pg/ml), no effect with one E_2 capsule replacement (circulating E_2 levels: 23.8 ± 2.0 pg/ml), and a stimulatory effect with two E_2 capsules

replacement (circulating E_2 levels: 36.8 ± 2.4 pg/ml). These various effects have been individually described in previous studies (Navarro *et al.* 2009a, Corander *et al.* 2010, Navarro *et al.* 2011a, Kinsey-Jones *et al.* 2012), but this is the first time where all three effects have been shown in a single study. It suggests that the effects of NK3R signalling on LH release are dependent on critical circulating levels of the sex steroid. In this respect, previous studies have yielded conflicting results, with some showing the reversal from an inhibitory to a stimulatory effect of NK3R agonism on LH secretion in OVX rats after E_2 treatment (equivalent to E_2 levels of proestrus), whereas others displayed the inhibitory effects without or with E_2 treatment (equivalent to E_2 levels of diestrus) (Navarro *et al.* 2011a, Grachev *et al.* 2012a). It has been shown in the present study that different regiments of E_2 replacement produced significant differences in circulating E_2 levels (see section 4.4.5). However, the differences of circulating levels of E_2 in the three replacement regimes used in the present study were very subtle. This may provide an explanation for the differences observed among previous studies: subtle variations in the circulating gonadal steroid milieu among studies could result in significant differences of LH secretion in response to NK3R agonism.

Numerous studies investigating the underlying mechanisms for the stimulatory effects of NKB on LH release have implicated the Kiss1-Kiss1r signalling system. The stimulatory effect of NK3R agonism on LH release in OVX rats treated with E_2 replacement is associated with up-regulated activities of Kiss1

neurones in the ARC (Navarro *et al.* 2011a). In ovary-intact prepubertal female rats, NK3R agonism induced LH release is dependent on the Kiss1r (Grachev *et al.* 2012b). Similar results have been found in prepubertal monkeys (Ramaswamy *et al.* 2011). Therefore, NK3R agonism might activate the Kiss1 to stimulate GnRH release.

The mechanisms underlying the inhibitory effects of NK3R agonism on LH release in hypoestrogenic conditions are less clear. Morphologic evidence indicated that NKB might directly influence GnRH secretion at the level of the ME, since close apposition of NKB GnRH fibres are observed in the ME and GnRH neurones express NK3R (Krajewski *et al.* 2005). However, intravenous administration of NK3R agonists, which should theoretically reach the ME, did not suppress the pulsatile release of LH in OVX rats replaced with low levels of E_2 (Grachev *et al.* 2014b). Therefore, the inhibitory effect of NK3R agonism might involve indirect routes via intermediary signalling systems. Dyn is a well-established inhibitory effector on LH release and is co-expressed with NK3R in the ARC neurones (Burke *et al.* 2006). The inhibitory effect of NK3R agonism on pulsatile LH release in OVX rats treated with low levels of E_2 is blocked by Dyn receptor antagonism in the ARC (Grachev *et al.* 2012a). Therefore, ARC Dyn signalling appears to regulate the inhibition of LH release by ARC NK3R agonism. However, the expression of Dyn receptor mRNA have not been detected in GnRH neurones (Mitchell *et al.* 1997, Sannella and Petersen 1997). Therefore, Dyn might not be the only intermediary signalling

system that regulates the inhibitory effect of NK3R agonism. Other unknown signalling systems that express Dyn receptor will have to relay the effect of Dyn to GnRH neurones.

A model has been presented in which NKB is probably the upstream regulator of GnRH release; with stimulation via Kiss1 and inhibition via Dyn signalling (Navarro *et al.* 2011a, Grachev *et al.* 2014b). This suggests that within a low-E_2 environment (such as that in OVX rats), activation of NK3R recruits a Dyn signalling mechanism to suppress the LH pulses, whereas in ovary-intact prepubertal female rats and diestrus adult female rats, activation of NK3R stimulates the release of Kiss1 to increase LH release. The present study further suggests that an expected transitional stage might exist between these two stages, as activation of NK3R did not affect the LH release in OVX rats replaced with middle levels of E_2, i.e. replaced with one E_2 capsule. The underlying mechanisms for this non-responsive stage remain unclear. We speculate that this might be due to the opposing effects of both inhibitory and stimulatory of NK3R agonism on LH release, or an unresponsive state of the NK3R at that particular level of E_2.

The triphasic effects of NK3R agonism on LH release are reminiscent of activation of NK1R on LH release, since previous studies have shown that NK1R activation with substance P induced inhibitory, null or stimulatory effects on LH release in a variety of experimental paradigms (Vijayan and McCann 1979, Arisawa *et al.* 1990, Kalra *et al.* 1992). Therefore, the effects of NK1R

agonism on LH release might similarly be modulated by the circulating levels of E_2. Indeed, the present study has shown that activation of NK1R in the ARC induced inhibitory effects on LH release in OVX rats without E_2 replacement, while stimulated LH release in OVX rats replaced with two E_2 capsules (see Chapter 5). This indicated that the biphasic effects of NK1R activation on LH release are dependent on the circulating levels of E_2. Although we did not study the effects of NK1R agonism on LH release in OVX rats replaced with single E_2 capsule, it is reasonable to speculate that a similar transition stage might exist between stimulation and inhibition.

Since we administered the NK1R agonists directly into the ARC, this indicates that the biphasic effects of NK1R agonism on LH release are probably mediated in this brain region. Since the expression of *Tacr1* (encoding NK1R) mRNA has been found in ARC Kiss1 neurones (Navarro *et al.* 2015), it is possible that activation of NK1R in the ARC is modulated through the ARC KNDy neurones. The stimulatory effects of substance P on LH release are indicated to be dependent on the Kiss1r signalling system. It has been shown that *Kiss1r* knockout mice nullified the stimulatory effects of NK1R agonism on LH release (Navarro *et al.* 2015). It was also shown in previous studies that substance P can directly increase the excitability of mouse ARC Kiss1 neurones (de Croft *et al.* 2013). Taken together, it is possible that NK1R agonists activate the ARC Kiss1 neurones to induce LH release, just like NK3R activation. However, the underlying mechanisms for the inhibitory effects of NK1R agonism on the

pulsatile LH release are not understood. Intra-ARC administration of NK3R agonists induced inhibition of the pulsatile LH release and is modulated by the Dyn (Grachev *et al.* 2012a). Since the NK1R is also expressed on KNDy neurones, NK1R agonism might also activate the ARC Dyn signalling to inhibit the pulsatile LH release. Indeed, it has been shown that substance P and NKB can activate KNDy neurones with similar fashions (de Croft *et al.* 2013). More studies will be needed to address this speculation.

Both Kiss1 and GnIH/RFRP-3 belong to the peptide family named RFamide peptides, which has been characterised as a major regulator of GnRH neurones (Parhar *et al.* 2012). Kiss1 plays an important role in stimulating the HPG axis, whereas GnIH/RFRP-3 has been shown to inhibit various aspects of reproductive functions, including the gonadotrophin release (Kriegsfeld *et al.* 2006, Johnson *et al.* 2007). However, previous studies have not established the role of RFRP-3 in the regulation of pulsatile LH release. It has been shown in the present study that icv administration of RFRP-3 dose-dependently inhibited LH pulse frequency in OVX rats (see section 6.4.1). This suggests that the inhibitory effects of RFRP-3 on LH release are through inhibiting the GnRH pulse generator. Therefore, RFRP-3 might be able to regulate the KNDy signalling systems in the ARC. However, intra-ARC administration of RFRP-3 did not affect the pulsatile LH release in OVX rats (see section 6.4.6). This indicates that RFRP-3 signalling is not capable of affecting the ARC KNDy neurones directly. Recent studies suggest that 25% of ARC Kiss1 neurones

express GPR147, the receptor for RFRP-3, and 35% of ARC Kiss1 neurones receive fibre inputs from RFRP neurones (Poling *et al.* 2013). However, it is not known what percentage of Kiss1 neurones both express GPR147 and are innervated by RFRP-3, which casts doubt on the functional importance of the above morphological observations. Taken together, these data do not support the postulate that RFRP-3 directly acts on the ARC KNDy neurones to affect the pulsatile LH release. Nevertheless, endogenous RFRP-3 signalling has an inhibitory effect on ARC Kiss1 neurones, since the number of *Kiss1* mRNA expressing neurones in the ARC increased by 60% in *GPR147* knockout mice (Leon *et al.* 2014). Therefore, intermediate signalling systems might exist to relay this inhibitory effect. It has been shown in the present study that the suppression of pulsatile LH release by icv administered RFRP-3 can be partially blocked by icv administered μ-opioid receptor antagonist (see section 6.4.5). Therefore, μ-opioid receptor and its endogenous ligand might function as the intermediate signalling systems. β-endorphin, one of the endogenous ligand for the μ-opioid receptor has been shown to mediate effects on various reproductive functions, including the inhibition of sexual behaviour and gonadotrophin release (Pfaus and Gorzalka 1987, Bonavera *et al.* 1993). It has been shown that central administration (icv) of β-endorphin can inhibit the pulsatile release of LH in castrated male rats (Kinoshita *et al.* 1980). Therefore, the inhibitory effects of RFRP-3 on pulsatile LH release might be mediated via β-endorphin neurones. Indeed, it has been shown that β-endorphin neurones are located in the ARC in

rats (Finley *et al.* 1981), where both RFRP-3 receptor and fibres have been found (Kriegsfeld *et al.* 2006, Poling *et al.* 2013). Future studies will be needed to investigate whether the ARC β-endorphin neurones co-express with GPR147 and are innervated by RFRP-3 fibres. Furthermore, β-endorphin neurones might not directly innervate ARC KNDy neurones since very moderate levels of μ-opioid receptors mRNA have been detected in the ARC (Mansour *et al.* 1995). Other intermediate signalling systems that express μ-opioid receptors might further relay the effects from β-endorphin to the KNDy neurones. Indeed, β-endorphin fibres have been found in many neuroendocrine important regions including the mPOA, the bed nucleus of the stria terminalis (BNST) and MeA, where dense expression of μ-opioid receptors have been found (Finley *et al.* 1981, Mansour *et al.* 1995). Taken together, icv administered RFRP-3 may affect the GnRH pulse generator indirectly via multiple intermediate signalling systems such as the β-endorphin signalling.

It has been shown in the present study that the antagonism of Kiss1r in the MeA reduced the pulsatile LH release in OVX rats (see section 7.4.1). The expression of *Kiss1* mRNA in the MeA can be affected by the stage of oestrous cycles in rats, as well as the levels of sex steroids in both sexes (Kim *et al.* 2011). Therefore, the MeA Kiss1-Kiss1r signalling may play an important role in regulating the reproductive axis. Previous studies have implicated the MeA in the regulation of the LH release, although the data are controversial. Both MeA lesioning and stimulation have been shown to induce LH secretion in rats

(Lawton and Sawyer 1970, Beltramino and Taleisnik 1978). Other studies have shown that lesions of the MeA had no effect on LH release in rodents (Eleftheriou *et al.* 1970, Docke *et al.* 1983). These conflicting data might be due to the different experimental paradigms used. Nevertheless, these studies only measured mean circulating levels of LH. A more recent study monitoring LH pulses has shown that lesioning of MeA has no effects on the pulsatile LH release in OVX rats (Lin *et al.* 2011). However, lesioning of the MeA blocks restraint stress-induced suppression of pulsatile LH release (Lin *et al.* 2011). This suggests that the MeA Kiss1 *per se* is not involved in the generation of LH pulses, but mediates, at least in part, the suppression the GnRH pulse generator frequency in response to psychological stressors. Hypothalamic Kiss1 signalling has been implicated in the psychological stress-induced suppression of the GnRH pulse generator. Restraint stress reduced the expression of *Kiss1* mRNA in both the mPOA and the ARC, which was associated with an inhibition of pulsatile LH release (Kinsey-Jones *et al.* 2009). It is plausible that psychological stressors might also affect Kiss1-Kiss1r signalling in the MeA, although further studies will be required to establish this. It has been shown in the present study that the blockade of Kiss1-Kiss1r signalling in the MeA reduced the frequency of LH pulses, which is in keeping with the postulate that psychological stress might down-regulate Kiss1-Kiss1r signalling in this limbic brain structure to suppress the GnRH pulse generator. The underlying mechanisms for the MeA Kiss1-Kiss1r signalling in the regulation of GnRH pulse generator activity are

unknown. It has been shown that the MeA projects directly to the ARC where the GnRH pulse generator is located (Coolen and Wood 1998, Usunoff *et al.* 2009). However, these projections are very sparse, indicating a rather limited level of interaction between these brain regions. Alternatively, MeA may affect other brain regions that regulate the GnRH pulse generator. It has shown that MeA has extensive direct projections to the mPOA and the BNST (Canteras *et al.* 1995, Coolen and Wood 1998), both of which have been implicated in regulating the pulsatile GnRH release. Intra-mPOA administration of a wide range of neuropeptides, including CRF, GABA and calcitonin gene-related peptide, suppresses pulsatile LH release (Jarry *et al.* 1991, Li *et al.* 2009a, Kinsey-Jones *et al.* 2010). Therefore, an unknown neurocircuit relay via the mPOA to the ARC to modulate the GnRH pulse generator might exist. Likewise, it has been shown that intra-BNST administration of noradrenaline and CRF inhibit the pulsatile LH release (Yamada *et al.* 2006, Li *et al.* 2011). Furthermore, extensive projections have been found from the BNST to the ARC (Dong and Swanson 2006). Taken together, the mPOA and the BNST might relay the effects of MeA Kiss1-Kiss1r signalling to the GnRH pulse generator in the ARC.

In summary, the main topic of the studies presented in this thesis invovles the role of various neuropeptides in regulating the pulsatile release of GnRH (Fig. 8.1) which is essential for initiating puberty and maintaining normal reproductive functions. The core of this regulation is the KNDy neurones in the ARC, which are thought to represent part of the neural construct of the GnRH

pulse generator. KNDy neurones express NK3R and project to each other to form an interconnected network. The synchronized activation of KNDy neurones via NKB-NK3R signalling may produce pulsatile release of Kiss1 at the level of the mPOA and/or the ME to activate GnRH neurones to release GnRH pulses. Other neuropeptide signalling systems may directly or indirectly act on KNDy neurones to affect the pulsatile release of GnRH. KNDy neurones express NK1R, the receptor for substance P which may therefore act directly on KNDy neurones in the ARC to alter LH pulse frequency. RFRP-3 neurones found predominantly in the DMH are known to directly project to the KNDy neurones. However, a direct action of RFRP-3 on the KNDy neurones was not observed in present studies, in terms of altered LH pulse frequency, which suggests that other unknown signalling system may mediate the suppressive effect of RFRP-3 on the pulsatile release of GnRH. Finally, Kiss1 signalling in the MeA profoundly modulates the pulsatile release of GnRH, although the neurochemical phenotype and trajectory of the functional anatomical connections between the MeA and the mPOA and/or the ARC remain to be established.

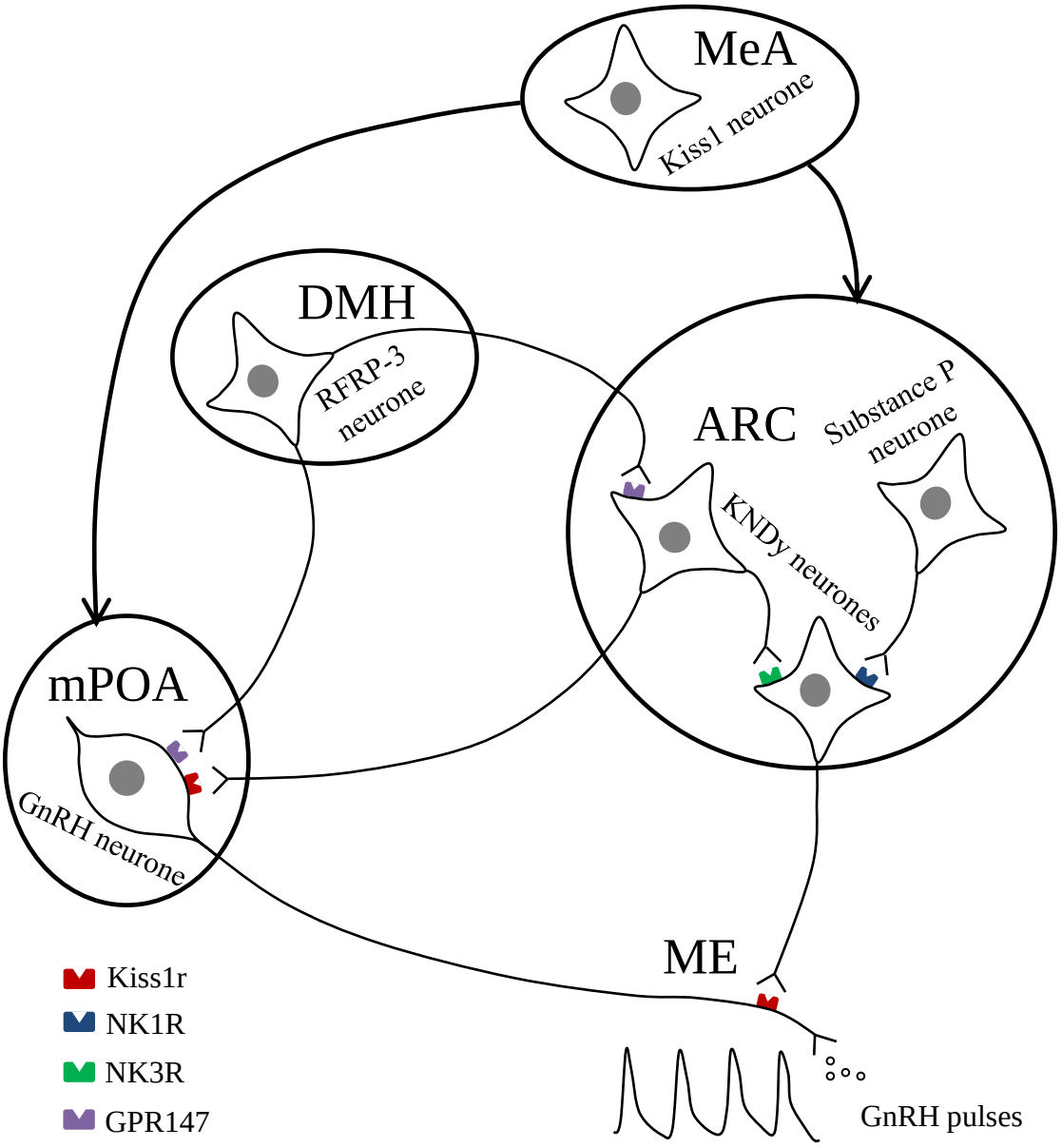

Fig. 8.1 Schematic diagram demonstrating the neural signalling systems regulating the pulsatile release of GnRH. The ARC KNDy neurones functionally interconnect with each other via NK3R, and project to GnRH neurones cell bodies in the mPOA and terminals in the ME. Kiss1 activates GnRH neurones via Kiss1r to release GnRH pulses at the level of the ME. Substance P may act on the KNDy neurones via NK1R. Furthermore, RFRP-3 neurones in the DMH may act on KNDy neurones and/or GnRH neurones via GPR147. However, the present study suggests that other unknown signalling systems may mediate the suppressive effect of RFRP-3 on pulsatile release of GnRH. Finally, Kiss1 signalling in the MeA modulates pulsatile release of GnRH possibly via neural projections, of unknown neurochemical phenotype, from the MeA to the mPOA and/or the ARC.

FUTURE STUDIES

The preceding experimental work has answered a number of important questions regarding the role of Kiss1, NKB, substance P and RFRP-3 in regulating the timing of puberty onset and LH release. The future directions of work which could follows on from the present study include:

1. Elucidating the mechanisms underlying the timing of puberty onset in over-nutritional status.

Is Dyn involved in the regulation of the pulsatile LH release and timing of puberty onset in over-nutritional status?

In the present study, we have shown that NK3R antagonism delayed the puberty onset and decreased the frequency of pulsatile LH release in female rats fed with high fat diet. We speculated that this may reflect the model that oscillation of NKB/Dyn in the ARC controls the pulsatile mode of GnRH release, which regulates the timing of puberty onset in over-nutritional status. However, little is known about the role of Dyn in the regulation of puberty onset in over-nutritional status. To address this, prepubertal female rats fed with a high fat diet should be chronically administered (icv) with Dyn. Furthermore, the timing of puberty onset and the dynamic of LH release should be examined in these rats.

2. Elucidating the neuronal pathways involved in the substance P-induced suppression of the GnRH pulse generator.

Is Dyn involved in the suppression of pulsatile LH release induced by intra-ARC administered substance P?

In the present study, we have shown that intra-ARC administration of substance P can suppress the pulsatile release of LH in OVX rats without E_2 replacement. However, the underlying mechanisms are still unknown. It is speculated that, similar to NKB, the inhibitory effects on pulsatile LH release by substance P are modulated by the ARC Dyn signalling. To address this, Dyn receptor antagonist should be directly administered into the ARC and the suppressive effect of substance P on pulsatile LH release assessed.

3. Elucidating the role of the MeA Kiss1 in regulating the sexual maturation.

Does the MeA Kiss1 affect the timing of puberty onset and the pulsatile LH release?

It is well-established from lesion studies that the MeA affect the timing of puberty onset in female rats. The MeA Kiss1 might be involved in this process. We have shown that intra-MeA administration of a Kiss1r antagonist suppressed the pulsatile release of LH. However, it is unknown whether the MeA Kiss1 can

also affect the GnRH pulse generator during the sexual development. To address these questions, prepubertal female rats should be chronically administered with Kiss1r antagonist into the MeA. The effects on pubertal timing and dynamic of LH release should be examined.

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